EUKARYOTIC CELL, Feb. 2010, p. 306–314 Vol. 9, No. 2
1535-9778/10/$12.00 doi:10.1128/EC.00248-09

tRNA Gene Sequences Are Required for Transcriptional Silencing in *Entamoeba histolytica*▽

Henriette Irmer,* Ina Hennings, Iris Bruchhaus, and Egbert Tannich

Bernhard Nocht Institute for Tropical Medicine, Bernhard Nocht Str. 74, 20359 Hamburg, Germany

Received 27 August 2009/Accepted 9 December 2009

Transcriptional silencing by *trans* inactivation can contribute to the regulation of gene expression in eukaryotic cells. In the human intestinal protozoan parasite *Entamoeba histolytica*, *trans* inactivation of the amoebapore-A gene (*AP-A*) was recently achieved by episomal transfection of *E. histolytica* trophozoites with the plasmid psAP1. The mechanism of *AP-A trans* inactivation is largely unknown, though it was suggested that a partial short interspersed transposable element (SINE) is required. By systematic assessment of various *E. histolytica* isolates transfected with psAP1 derivates, *trans* inactivation of *AP-A* was restricted to the strain HM-1:IMSS (2411) but could not be achieved in other standard laboratory strains. Importantly, sequences of an *E. histolytica* tRNA array that were located on psAP1 in close proximity to the *AP-A* upstream region and comprising the glutamic acid (TTC) (E) and tyrosine (GTA) (Y) tRNA genes were indispensable for *AP-A* silencing. In contrast to the case described in previous reports, SINE was not required for *AP-A trans* inactivation. *AP-A* expression could be regained in silenced cells by episomal transfection under the control of a heterologous *E. histolytica* promoter, opening a way toward future silencing of individual genes of interest in *E. histolytica*. Our results indicate that tRNA gene-mediated silencing is not restricted to *Saccharomyces cerevisiae*.

The formation of heterochromatin is one of several hierarchical levels in the regulation of gene expression in eukaryotic cells (reviewed in reference 40). The expression of genes can be repressed by histone modifications shifting the chromatin state to heterochromatin. Telomeric, centromeric, and repetitive sequences are part of the heterochromatin (15). Heterochromatin formation and the architecture of the nucleus are closely interrelated. Some domains of eukaryotic cell nuclei are transcriptionally active, and others, such as the nuclear periphery, which is often organized as heterochromatin, are silent. The position of a gene within the nucleus can determine its expression state, also known as position effect. For a gene to become silenced, it is sufficient for it to be located in close proximity to the periphery of the nucleus (3).

Shifting of a gene locus into the state of inactive chromatin can be mediated by DNA elements, which are not part of the gene locus (in *trans*). Such events of *trans* inactivation (also termed transvection) in several eukaryotic organisms have been described, but the mechanism is poorly understood. It was shown that in mice, contact with centromeric heterochromatin can result in spreading of chromatin in *trans* (7, 14). *trans* inactivation of the *Drosophila melanogaster brown* locus is mediated by a large insertion of heterochromatin in one allele that silences a second allele as well (10, 35). Genes close to the *brown* locus are also silenced. *trans* inactivation appears to be enhanced by a stretch of endogenous sequence of about 300 bp near the heterochromatin insertion. The 300-bp element contains a bidirectional promoter and potential binding sites for silencing proteins that may influence heterochromatin formation (34).

Several sequence elements and proteins have been identified as being involved in heterochromatin silencing (8, 31). The HM mating-type loci in *Saccharomyces cerevisiae* contain silencer elements, termed E and I. Some silencer elements have been described to be functional as autonomous replication sequences, and some autonomous replication sequences can function as silencers (13, 16). tRNA genes have been shown to exhibit opposing effects at the silent HM loci in *S. cerevisiae*. Depending on the mating type, they can act as boundary elements that block the spread of heterochromatin (30) or as transcriptional repressors of genes transcribed by polymerase II (21, 24, 37). This mechanism is referred to as tRNA gene-mediated gene silencing (tgm). tgm depends on nucleolar localization and active transcription of the tRNA (41).

The human intestinal protozoan parasite *Entamoeba histolytica* is poorly studied with respect to gene expression and regulatory mechanisms. Although the principal nuclear organization has not been characterized in detail, certain characteristics that are distinct for this organism have been reported. These includes the presence of ribosomal DNA (rDNA) on episomes that are located in the vicinity of the nuclear membrane (20, 43). In addition, unique arrangements of tRNA genes have been described previously (9). tRNA genes are organized as multiple tandem-array units, which are spaced by tandem repeats of AT-rich sequences. There exists evidence that these arrays are located at chromosome ends and may be functionally equivalent to telomeres, which are absent in *E. histolytica* (28). It was suggested that tRNA arrays play a role in the structural organization of the nucleus (9).

In order to develop a tool to shut down gene expression in *E. histolytica*, we systematically assessed *trans* inactivation of the amoebapore-A (*AP-A*) gene. *AP-A* constitutes one of the main pathogenicity factors of this parasite and is involved in host

* Corresponding author. Present address: Grisebachstr. 8, Institute of Microbiology and Genetics, Department of Molecular Microbiology and Genetics, Georg-August-University, Göttingen, 37077 Göttingen, Germany. Phone: 49 551 393777. Fax: 49 551 393330. E-mail: hirmer@gwdg.de.

▽ Published ahead of print on 18 December 2009.

tissue destruction (25). We followed up on a previous study reporting successful silencing of the endogenous *AP-A* gene after transfection with the plasmid psAP1 bearing an *AP-A* expression cassette (5). The 5′ region of the *AP-A* cassette was required for silencing, whereas the *AP-A* open reading frame (ORF) and the 3′ end were not. An RNA interference-based mechanism was highly unlikely, since small interfering RNAs were not detected (2). However, it was suggested that the presence of a 140-nucleotide fragment, representing part of a short interspersed transposable element (SINE) together with an adjacent thymidine-rich (T-rich) region on the plasmid is required to induce silencing (2). The endogenous, complete SINE is located approximately 300 bp upstream of the *AP-A* open reading frame in the reverse orientation. Whether SINE can indeed mechanistically underlie silencing is under debate. Recently, *trans* inactivation of further genes in *E. histolytica* was achieved but only in cells in which *AP-A* was already silenced (4, 6, 32). Here, we report for the first time that AP-A expression can be reconstituted in AP-A-deficient amoebae, opening a way toward silencing of genes of interest without additional AP-A deficiencies. We also show that SINE and the T-rich region are dispensable for *AP-A trans* inactivation. Instead, the *trans* inactivation property could be assigned to two elements present in close proximity on psAP1, namely, an array of the *E. histolytica* $Glu^{(TTC)}$ (E) and $Tyr^{(GTA)}$ (Y) tRNA genes and a 213-nucleotide fragment of the *AP-A* 5′ region. *trans* inactivation was dependent on the presence, but not the orientation, of the two elements. To our knowledge this is the first report of tRNA gene-mediated transcriptional repression of gene expression in *trans*.

MATERIALS AND METHODS

Strain and culture conditions. *Entamoeba histolytica* trophozoites were cultured axenically in plastic culture flasks with TYI-S-33 medium (11). The culture medium was changed every other day. Single-cell cloning of amoebae was performed by limiting dilution in 24-well culture plates under anaerobic conditions by using Anaerocult A (Merck). Harvesting of cultured amoebae was carried out with cells in the late logarithmic phase of growth by chilling on ice for 10 min and sedimentation at 430 × *g* at 4°C for 5 min.

Plasmid construction and transfection. Plasmid psAP1Δ2600 was generated by removing a 2,618-bp XbaI fragment from silencing plasmid psAP1 (5). psAP1Δ2600, which lacks the glutamic acid (TTC) and tyrosine (GTA) (EY) tRNA array, a partial tetracycline (Tet) repressor sequence, and part of the *E. histolytica* actin gene 3′ sequence that is flanking the neomycin resistance gene, was used as backbone for generating deletion mutant plasmids. PCR-amplified fragments bearing different fragments of the EY tRNA array (Table 1; see Fig. 4) were cloned into the TOPO TA vector (Invitrogen), sequenced, and directionally subcloned into the XbaI and NotI restriction sites of psAP1Δ2600. Cloning of the terminator site into psAP1 was achieved by oligonucleotide cloning into the BamHI/NotI site. Two plasmids were used as target vectors to generate mutants with mutations and deletions of the *AP-A* 5′ end. In both psAP1 and psAP1Δ2600 EY600, the *AP-A* cassette was removed by digestion with SacII, and the vector was religated. Subsequently, PCR-amplified *AP-A* 5′ fragments (Table 1) were cloned into the NotI restriction site. pNAP-A was derived from the *E. histolytica* transfection vector pN (neocassette) (19) by introducing the *AP-A* cassette into the HindIII-XbaI sites. The plasmid pNinAP-A was derived from the pN vector bearing the *Lec485* promoter and the *ehrpl27a* intron upstream of the *AP-A* ORF. The intron was cloned into the KpnI-BamHI site of pN, and an NheI site was introduced. The *AP-A* ORF was added by using the BamHI-NheI site. All plasmids were verified by molecular sequencing. Transfections were performed as described previously (19). psAP1 background cells (AP-A-silent cells) were grown without the neomycin analogue G418 for at least 4 months, and G418 sensitivity was verified before transfection. Drug selection was started 48 h after transfection by using 10 μg/ml of G418. Total RNA was harvested at least 3 weeks posttransfection. The presence or absence of the transfected plasmid was analyzed by PCR.

tRNA mutagenesis. Site-directed mutagenesis was carried out using the Expand long-template PCR system (Roche) by using specific sense and antisense primers encompassing the nucleotides to be mutated (Table 1). The plasmid EY1000 was used as the template (30 ng). Amplification conditions were 30 cycles of 1 min at 95°C, 1 min at 50°C, and 10 min at 65°C. Restriction digestion was performed with DpnI at 37°C. A 7-μl volume of the reaction mixture was used for transformation into *Escherichia coli* DH5α cells. The relevant fragments were validated by molecular sequencing and subcloned into psAP1 with XmaI and BamHI.

Northern blotting. RNA was isolated from cultured amoebae by using RNeasy (Qiagen). For Northern blotting, 10 μg of total RNA was separated on agarose gels and transferred to nylon membranes. Blots were hybridized with radiolabeled DNA probes. Hybridizations were performed in Dig Easy Hyb (Roche) at 42°C. Blots were washed with 2× and 1× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7) at 55°C.

Immunoblotting of trophozoite extract. Frozen trophozoites (4.5×10^9 cells) were thawed in the presence of the proteinase inhibitor E-64 [100 μM, *trans*-epoxysuccinyl-L-leucylamino-(4-guanodino)butane]. The cells were frozen and thawed in three cycles in dry ice and stored at −70°C. Tricine-SDS-PAGE was performed under reducing conditions by using 13% separation gels (36). Subsequent immunoblotting was carried out as described previously (25). Briefly, wet blotting was used with 25 mM Tris, 192 mM glycine, 1.3 mM SDS (pH 8.3)–20% methanol as the transfer buffer onto a nitrocellulose membrane (BA83 Protan; Schleicher and Schuell). Antisera to amoebapores A and B were kindly provided by Matthias Leippe and used in dilutions of 1:300 and 1:5,000, respectively (25). Detection was carried out using luminol (Sigma) and *para*-hydroxycoumaric acid in the presence of H_2O_2.

RT-PCR and quantitative PCR. DNase digestion and subsequent cDNA synthesis were carried out in duplicate for each sample by using 1 μg of RNA with the QuantiTect reverse transcription kit (Qiagen). Amplification was performed in a Rotor-Gene (Corbett) instrument with the QuantiTect SYBR green kit (Qiagen) by using 1 μl of the cDNA and *E. histolytica AP-A*, *actin*, or tRNA gene primers (Table 1). Amplification conditions were as follows: 50 cycles of 15 s at 95°C, 20 s at 65°C, 20 s at 68°C, and an adjacent melting step (42 to 95°C). The amount of *AP-A* relative to *actin* RNA was quantified using the ΔΔ*CT* method provided in Rotor-Gene software 6.0.14 (27). All quantitative reverse transcription-PCR (qRT-PCR) experiments were performed at least in triplicate.

RESULTS

Transcriptional silencing of the *E. histolytica* amoebapore-A gene is restricted to a particular amoeba strain. In order to examine whether transcriptional silencing of the amoebapore-A gene(*AP-A*) can be achieved in all *E. histolytica* strains or whether it is restricted to particular cell lines, various amoeba isolates were transfected with the silencing plasmid psAP1 as reported previously (5). Northern blot analysis revealed that *AP-A trans* inactivation was achieved only in strain HM-1:IMSS (2411) (Fig. 1), kindly provided by David Mirelman, but not in other standard laboratory strains from the American Type Culture Collection (ATCC), such as another HM-1:IMSS strain (no. 30459), 200:NIH (no. 30458), and HK9 (no. 30015). In contrast, transfection of psAP1 into amoeba isolates other than HM-1:IMSS (2411) resulted in a substantial increase of *AP-A* mRNA abundance relative to that of mock-transfected cells (Fig. 1). PCR analysis of the various amoeba isolates by using highly polymorphic markers suitable for subtyping individual strains (1) showed identical DNA patterns between the ATCC HM-1:IMSS strain and HM-1:IMSS (2411), indicating that the two strains are indeed closely related (data not shown). In addition, sequencing of the entire *AP-A* locus did not reveal any differences between the isolates. We note that *AP-A trans* inactivation in HM-1:IMSS (2411) was not detectable before day 18 posttransfection. Interestingly, *trans* inactivation did not require long-term selection for the transfected plasmid. *AP-A trans* inactivation was obtained

TABLE 1. Oligonucleotides used for PCR amplification, oligonucleotide cloning, mutagenesis, and quantitative RT-PCR

Target/construct[a]	Oligonucleotide	Sequence[b]	Orientation[c]
EY1000	OH68	attctagaTTTTATACTAATAGATAGG	S
	OH71	atgcggccgcTAGTGGATCCGACCAACCGG	AS
EY600	OH69	attctagaTATAGTCATGGTAAATCC	S
	OH71	atgcggccgcTAGTGGATCCGACCAACCGG	AS
Y200	OH70	attctagaGAATAGAAACATATATAAGCAC	S
	OH71	atgcggccgcTAGTGGATCCGACCAACCGG	AS
E800	OH68	attctagaTTTTATACTAATAGATAGG	S
	OH72	atgcggccgcCCTTTTATATTTCTATATGTGC	AS
E500	OH68	attctagaTTTTATACTAATAGATAGG	S
	OH73	atgcggccgcGCTAAAAAATTCCATCGCCGGG	AS
Terminator in psAP1	OH80	gatccTTTTTTTTgc	S
	OH81	ggccgcAAAAAAAAg	AS
E500Δ terminator	OH68	attctagaTTTTATACTAATAGATAGG	S
	OH79	atgcggccgcTTCCATCGCCGGGAATCGAACCC	AS
pNAP-A EY1000	OH76	atctcgagggtacccgggatccGACCAACCGGATTCGAACCAGTGACC	S
	OH74	attctagaTAGTGGATCCGACCAACCGG	AS
pNinAP-A	OH13	atgctagcACAAACAATCATGAAAGCC	S
	OH14	atggatccTTAGCAAGCATGAATCTTAGC	AS
AP-A 5′	OH88	atgcggccgcGATTGTTTGTAAGATATG	S
	OH89	atgcggccgcCTTGCTGCACCCTTTG	AS
AP-A 5′ ΔSINE	OH101	atgcggccgctTTTATTATTTAAAAAACAAAATAG	S
	OH89	atgcggccgcCTTGCTGCACCCTTTG	AS
AP-A 5′ Δ240	OH116	atgcggccgcTTTAATAAATATTAAAAGAGAAG	S
	OH89	atgcggccgcCTTGCTGCACCCTTTG	AS
AP-A 5′ Δ260	OH117	atgcggccgcGAGAAGAAATGAAATAATCA	S
	OH89	atgcggccgcCTTGCTGCACCCTTTG	AS
AP-A for qPCR	ap-a s30	CACTAAGGGAGCTGATAAAGTAAAAGATTA	S
	ap-a as25	TCCAAAATCAAGAACTTTAGTGCAA	AS
Actin gene for qPCR	act s28	TGTAGATAATGGATCAGGAATGTGTAAA	S
	act as23	CAATGGATGGGAATACAGCTCTT	AS
Y tag1 for mutagenesis	OH227	CTCAGTTGGTAGAGCGGATGACTGTAGATGTAGAATAGA ATTCATTAGGTCACTGGTTCGA	S
	OH228	TCGAACCAGTGACCTAATGAATTCTATTCTACATCTACAG TCATCCGCTCTACCAACTGAG	AS
Y mutA	OH238	CCGACCATAGCTCAGTTTCTAGAGCGGATGACTG	S
	OH239	CAGTCATCCGCTCTAGAAACTGAGCTATGGTCGG	AS
Y tag1 for qPCR	OH225	CTCAGTTGGTAGAGCGGAT	S
Y	OH226	TCCGACCAACCGGATTC	AS
Y mutB1 for qPCR	OH245	TCCGACCAACCGGACTC	AS

[a] Δ240, deletion of 240 nucleotides; Δ260, deletion of 260 nucleotides; Y mutA and Y mutB1, Y gene mutants (see Fig. 5 legend).
[b] Lowercase letters represent restriction sites for subsequent cloning into the corresponding vectors for site-specific mutagenesis.
[c] S, sense; AS, antisense.

even after selection for less than a week and subsequent growth without further addition of G418, suggesting that psAP1 initially triggers *trans* inactivation but is not required to further maintain it. Transinactivated cells revealed growth kinetics similar to those of wild-type cells. In particular, AP-A-silenced amoebae did not have any growth advantage compared to nontransfected or mock-transfected cells. Consistent with previous observations (5), psAP1-transfected amoebae had not only AP-A but also AP-B silenced (Fig. 2).

AP-A expression can be reconstituted in *AP-A-trans* inactivated cells by episomal transfection. To investigate whether AP-A expression can be reconstituted in *AP-A*-transinactivated cells, an episomal transfection vector, pNinAP-A, was constructed. Transfection with this plasmid induces G418 resistance in *E. histolytica* trophozoites and contains the ORF of *AP-A*, flanked by the *E. histolytica lectin* gene promoter, an intron from a gene encoding an *E. histolytica* ribosomal protein (*rpl27a*), and *actin* 3′ sequences (Fig. 2A). The lectin gene promoter and the *AP-A* ORF are separated by an intron from the gene encoding an *E. histolytica* ribosomal protein (*rpl27a*). Transfection of pNinAP-A into *AP-A-trans* inactivated amoebae and subsequent selection with G418 led to significant expression of AP-A similar to that of wild-type cells. AP-A expression was dependent on the persistent presence of the expression plasmid, as omission of G418 selection resulted in a gradual loss of *AP-A* mRNA over time. Expression of amoebapore in pNinAP-A-transfected cells was restricted to AP-A, and expression of AP-B was not restored (Fig. 2). This intron from *rpl27a* was previously used in reporter gene experiments and seemed to have enhancer properties (unpublished data). Plasmid constructs containing no intron did not yield any AP-A reconstitution.

An *E. histolytica* tRNA array is required for *AP-A trans* inactivation. Initial transfection experiments using plasmid pNAP-A, which contains some sequence motifs similar to those of psAP1, increased *AP-A* mRNA expression but failed to induce *AP-A trans* inactivation in all amoeba strains examined, including HM-1:IMSS (2411) (Fig. 3A). Sequence comparison indicated that the two plasmids contain the same *AP-A* genomic fragment as well as the neomycin phosphotransferase

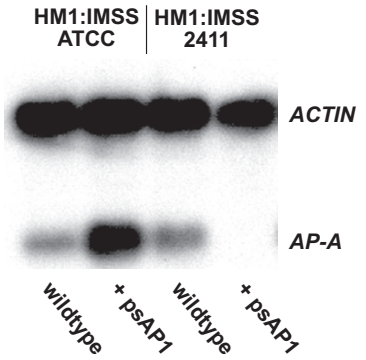


FIG. 1. *trans* inactivation of *AP-A* in the *E. histolytica* isolate HM-1:IMSS. The abundance of *AP-A* mRNA was analyzed in various *E. histolytica* isolates transfected with plasmid psAP1. Shown is an autoradiogram of a Northern blot from gel-separated total RNA isolated from nontransfected (wild-type) or psAP1-transfected amoebae of HM-1:IMSS (2411) or the ATCC HM-1:IMSS strain. The blot was sequentially hybridized with radiolabeled *AP-A* and *E. histolytica ACTIN* gene probes. Note that *trans* inactivation was achieved in HM-1:IMSS (2411) but not in other *E. histolytica* isolates.

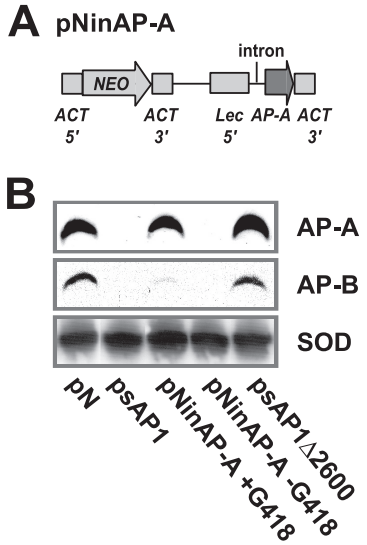


FIG. 2. Reconstitution of AP-A expression in *AP-A trans* inactivated cells by episomal transfection with plasmid pNinAP-A. (A) Schematic depiction of the expression vector comprising the neomycin resistance gene (*NEO*) under the control of *E. histolytica actin* (*ACT*) gene 5′ and 3′ sequences and the *AP-A* open reading frame (*AP-A*) under the control of *E. histolytica* lectin gene 5′ (*LEC* 5′) and *E. histolytica actin* gene 3′ (*ACT* 3′) sequences. The lectin gene promoter and the *AP-A* open reading frame are separated by an intron from the gene encoding *E. histolytica* ribosomal protein L27a. (B) Immunoblot analysis of *E. histolytica* extracts from various HM-1:IMSS (2411) transfectants tested with antisera against *E. histolytica* AP-A or AP-B. As a control, a parallel blot was developed with antiserum against *E. histolytica* superoxide dismutase (SOD). Extracts were obtained from the following transfectants: wild-type cells transfected with pN (neocassette vector) and selected with G418; wild-type cells transfected with psAP1; *AP-A*-silenced cells grown for several months without G418 and subsequently transfected with pNinAP-A and selected again with G418 (pNinAP-A+G418) or without G418 selection (pNinAP-A-G418); and wild-type cells transfected with psAP1Δ2600. Note that reconstitution of expression was achieved when selective pressure was maintained after transfection with pNinAP-A.

gene under the control of *E. histolytica actin* gene 5′ and 3′ sequences. However, compared with pNAP-A, psAP1 contained an extended *actin* 3′ sequence bearing additional *E. histolytica* genes (FUS1 and TFIIH) and a partial bacterial Tet repressor ORF followed by a stretch of amoebic sequence previously designated ARS. The latter has been shown to exhibit functional properties of autonomous replication sequences when transferred to a yeast artificial chromosome (29). However, careful analysis of the *E. histolytica* ARS revealed that it represents a tRNA array (9), comprising the glutamic acid (TTC) and tyrosine (GTA) tRNAs, separated by repetitive AT-rich elements (Fig. 3). To determine which of the psAP1-specific sequences are responsible for *AP-A trans* inactivation, a 2.6-kb fragment comprising part of the extended *actin* gene 3′ sequence and the bacterial Tet repressor ORF as well as the amoeba EY tRNA array was deleted (Fig. 3B). The resulting plasmid, psAP1Δ2600, was incapable of inducing *trans* inactivation of *AP-A* (Fig. 3A). Instead, psAP1Δ2600 transfection led to *AP-A* mRNA overexpression, as observed after transfection with pNAP-A (data not shown). However, when the 1.0-kb EY tRNA array sequence was reintroduced into psAP1Δ2600 or pNAP-A, both plasmids acquired *trans* inactivation capacity. The introduction of other *E. histolytica* tRNA arrays, such as LS or SPPCK (9), into psAP1Δ2600 did not induce *AP-A trans* inactivation (data not shown), suggesting that *trans* inactivation may be restricted to the EY tRNA sequence.

Glutamic acid (TTC) and tyrosine (GTA) tRNA genes are sufficient to mediate *AP-A trans* inactivation. In order to determine whether the complete tRNA array is required to mediate *AP-A trans* inactivation, a series of 5′ and 3′ deletion constructs was generated (Fig. 4A). In addition, the relevance of poly(T) stretches situated 3′ of amoeba tRNAs was investigated (Fig. 4B), and they most likely represent terminator sequences for polymerase III (Pol III) transcription as in other organisms. qRT-PCR analysis of amoebae transfected with the various constructs indicated that *AP-A trans* inactivation is independent of the presence or absence of the repeat elements or the poly(T) terminator sequences but requires the presence of both tRNA genes (Fig. 4). The orientation of the tRNA genes is not dependent on function (unpublished data).

Polymerase III transcription of the $tRNA^{Tyr}$ gene is not required for *trans* inactivation. To investigate whether polymerase III-dependent transcription of the episomal tRNAs is required for *trans* inactivation, a series of mutations was introduced into the $tRNA^{Tyr}$ gene of psAP1 (Fig. 5A). Interestingly, though some of the mutations had severe effects on the internal promoter regions and for tRNA transcription, none of the mutations altered *AP-A trans* inactivation capacity of the plasmid (Fig. 5B).

SINE is dispensable for *AP-A trans* inactivation. A SINE is located 5′ of the AP-A locus in the *E. histolytica* genome and thus is partially present on the psAP1 plasmid at the 5′ flanking region of AP-A. It has been suggested that the partial SINE sequence is required for the *trans* inactivation capacity of psAP1 (2). To further explore the characteristics of the 5′ flanking sequence required for *AP-A trans* inactivation, several 5′ deletions in the *AP-A* upstream region were performed in psAP1 plasmids (Fig. 6). Surprisingly, qRT-PCR analysis after

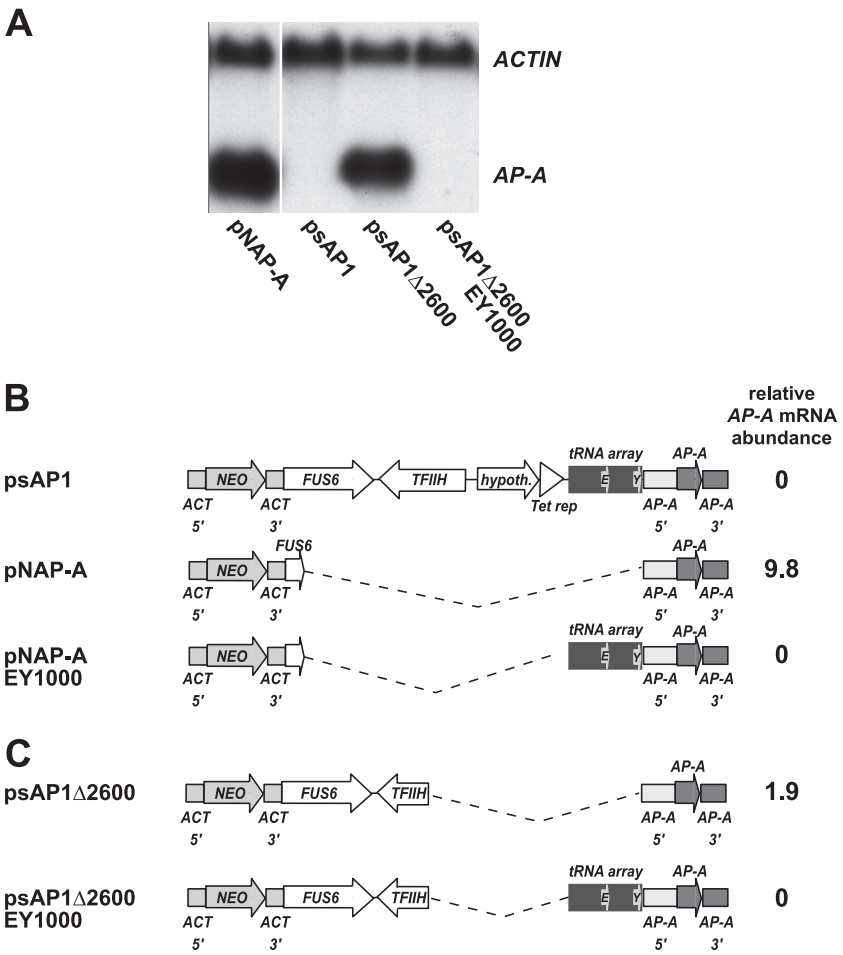


FIG. 3. An *E. histolytica* tRNA array is required to mediate *trans* inactivation of *AP-A*. (A) Northern blot analysis of gel-separated total RNA isolated from various amoeba transfectants as indicated. The blot was hybridized with radiolabeled *ACTIN* and *AP-A* gene probes. (B and C) Schematic depiction of sequence elements present on psAP1 and on various deletion constructs used for transfection. Compared to psAP1, plasmids pNAP-A and psAP1Δ2600 bear deletions of 3.2 and 2.6 kb, respectively, as indicated by the dotted lines. Transfection plasmids pNAP-AEY1000 and psAP1Δ2600EY1000 represent pNAP-A and psAP1Δ2600, respectively, in which the 1.0-kb EY tRNA array has been introduced. *AP-A* mRNA abundance was measured by qRT-PCR with primers specific for the *E. histolytica AP-A* and *actin* (*ACT*) genes, and the ΔΔ*CT* method was used for quantification. The mRNA abundance of wild-type HM-1:IMSS (2411) amoebae was set at 1. A value higher than 1 indicates overexpression, while a value of 0 indicates that *AP-A* was *trans* inactivated. Note that only those transfection plasmids containing the EY tRNA array have *AP-A*-transinactivating capacity. *NEO*, neomycin resistance gene. hypoth., hypothetical protein.

transfection of these constructs revealed that the deletion of SINE did not change the *trans* inactivation property of psAP1. Even constructs containing only 213 bp of the *AP-A* upstream region were capable of *trans* inactivation *AP-A* in transfected amoebae. However, shortening of the *AP-A* upstream region reduced the *trans* inactivation efficacy of the plasmid. As determined by single-cell cloning of transfected cells and subsequent qRT-PCT analysis, depending on the length of the 5′ flanking sequence, only about 20 to 50% of the cell clones were affected by *AP-A trans* inactivation. Thus, silencing became evident only after single-cell cloning but was not obvious in transfected bulk cultures.

DISCUSSION

To gain insight into the molecular mechanism of *trans* inactivation of *AP-A* in *E. histolytica* following transfection with the plasmid psAP1, we have identified two factors that are indispensable for *AP-A* transcriptional silencing. First, *AP-A* silencing is restricted to a particular cell line, and second, two specific tRNA genes are required on the plasmid in close proximity upstream of the *AP-A* promoter. In contrast to the case described by a previous report (2), a SINE motif located upstream of the *AP-A* promoter was not required. This discrepancy may be explained by our finding that shortening of the *AP-A* upstream region (e.g., by deletion of SINE) significantly reduces *trans* inactivation efficacy, as indicated by a reduced number of cells with an *AP-A*-silenced phenotype, which became evident only after single-cell cloning of transfected amoebae. The fact that the length of the *AP-A* upstream region plays a role for the *trans* inactivation capacity suggests that a base-pairing mechanism may be involved in *E. histolytica trans* inactivation.

Two sequence elements are required for *trans* inactivation, namely, the tyrosine and glutamic acid tRNA genes and about

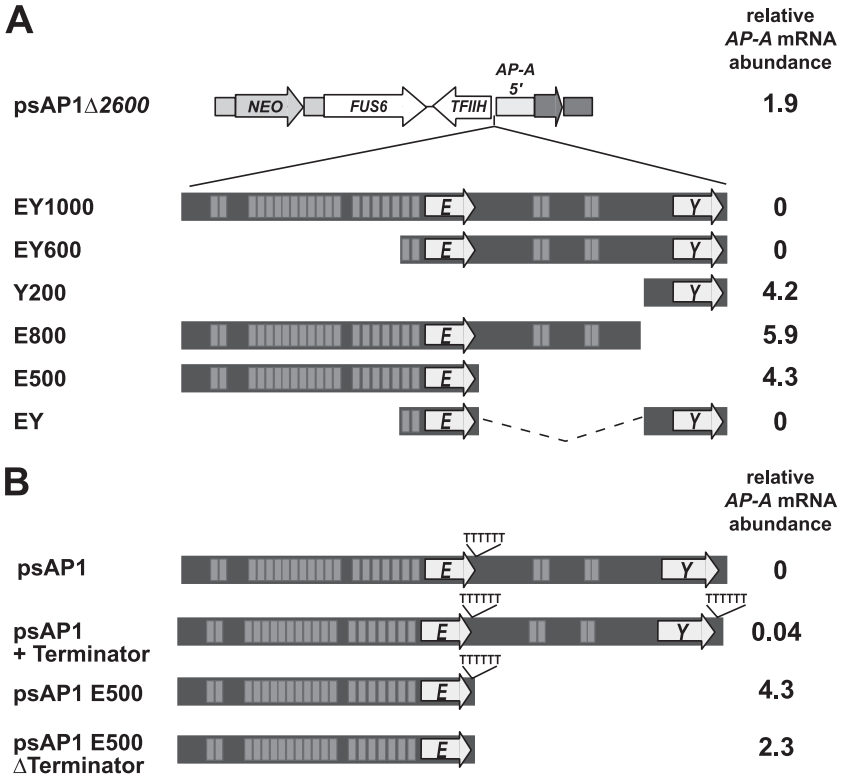


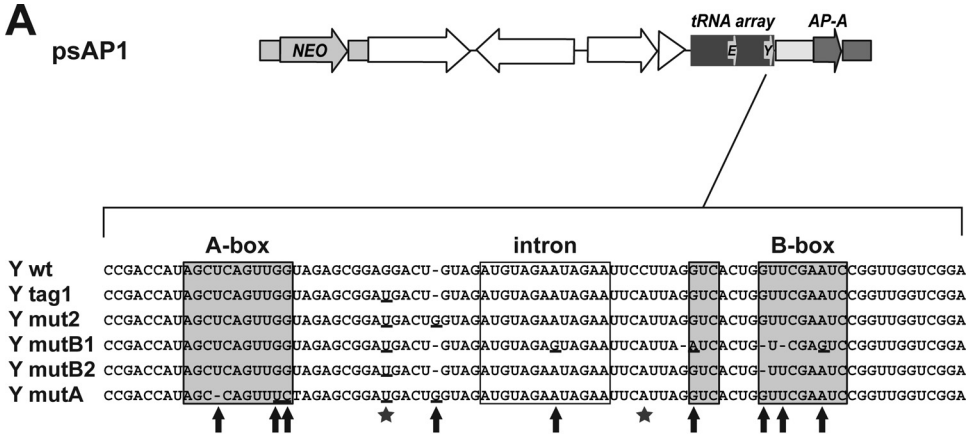
FIG. 4. Sequence motifs within the EY tRNA array are required for *AP-A trans* inactivation. (A) Transfection plasmid psAP1Δ2600, incapable of mediating *trans* inactivation of *AP-A*, was used as a backbone to introduce various modifications of the 1.0-kb EY tRNA array as indicated. Glutamic acid (E) and tyrosine (Y) tRNAs are represented by arrows, and AT-rich repeat elements are shown as dashes. *NEO*, neomycin resistance gene. (B) *AP-A* mRNA abundance was measured by qRT-PCR with primers specific for *AP-A* or actin and the $\Delta\Delta CT$ method for quantification. To the right, *AP-A* mRNA abundances relative to that of wild-type cells (set at 1) are given. The relevance of polythymidine stretches (TTTTT) adjacent to the tRNA sequences was investigated. Note that *trans* inactivation was observed only with constructs containing both tRNA sequences but was independent of the presence of poly(T) stretches or repeat elements.

200 bp of the *AP-A* 5′ region. These two elements operate in concerted action, as the EY tRNAs alone or in close proximity to other promoters cannot transinactivate, and neither does the *AP-A* promoter alone or in close proximity to other tRNA genes.

Two mechanisms found in other eukaryotes and which may play a role for the *trans* inactivation of *AP-A* in *E. histolytica* have been described previously. These are heterochromatic silencing in *trans* (transvection), as found in various organisms (12, 26, 33), and tgm, so far described exclusively for *S. cerevisiae* for tyrosine, leucine, and threonine (21). Both mechanisms depend on the nuclear architecture, and for both, it is likely that they play a role in *E. histolytica* transcriptional silencing. This does not exclude the possibility that other yet unknown factors may also participate in the silencing mechanism.

Chromatin structure. Attempts to localize tRNA genes by fluorescence *in situ* hybridization (FISH) experiments turned out to be difficult with *E. histolytica* (43). Hybridization was successful with a single EY tRNA probe (called M11) but was unsuccessful in colocalization experiments using rDNA and tDNA probes simultaneously.

Nevertheless, several observations support the concept that the AP-A locus and the plasmid psAP1 localize to the heterochromatic periphery. First, Anbar et al. showed that in *AP-A*-silenced cells, the *AP-A* gene is no longer associated with the methylated K4 residue of histone H3 (2). Second, some ARS sequences, such as the yeast 2μ origin, have been localized to heterochromatin and can act as silencers (16). The EY tRNA array has also been shown to have properties of autonomous replication sequences, at least when transferred to *S. cerevisiae* (29). Finally, the nuclear periphery, a heterochromatic region where telomeres are localized in other organisms, has been suggested to play a role in the silencing mechanism (39). As telomeres are absent in *E. histolytica*, it is believed that tRNA arrays are located at the end of the amoeba chromosomes, presumably at the nuclear periphery, and thus may play a structural role similar to that of telomeres (28). The organization of tRNA genes in *E. histolytica* is exceptional in that they form long arrays consisting of hundreds of tRNA sequences and AT-rich repeat elements (9). Hence, tRNA genes are organized as natural clusters in *E. histolytica* in contrast to *S. cerevisiae*, in which the spatially clustered tRNAs upon Pol III transcription have been shown to be colocalized at the nucleolus (reviewed in reference 17). Likewise, the nucleolus in *E. histolytica* is localized at the periphery, as demonstrated with rDNA episomes (20, 43) and recently with fibrillarin (23). With these results taken together, we propose that tRNA gene arrays and the tRNA genes of the plasmid might be colocalized at the nuclear periphery in *E. histolytica*. Further studies to



B

psAP1 variant	relative *AP-A* mRNA	Transcription of mutated tRNA
Y wt	0	NA
Y tag1	0	+
Y mut2	0	0
Y mutB1	0	0
Y mutB2	0	0
Y mutA	0	0

FIG. 5. The presence but not transcription of the tRNA on psAP1 is required for *AP-A trans* inactivation. (A) Schematic depiction of the relevant regions of psAP1 and location of various mutations introduced into the tyrosine (Y) tRNA gene to test the importance of the respective residues for the *trans* inactivation capacity of psAP1. Residues deviating from the wild-type sequence (Y wt) are underlined (Y mut). (B) Presence or absence of *AP-A* mRNA and tRNA transcripts after transfection with psAP1 containing mutations within the tyrosine tRNA gene. The abundances of *AP-A* mRNA and transcribed mutated tRNA relative to that of the wild type (set to 1), as determined by qRT-PCR, are given. Note that mutated tRNA was detectable only when mutations indicated by asterisks in panel A were introduced (Y tag1) but not when bearing mutations marked by arrows in that panel. The latter primarily comprised mutations located within the control region (A-box and B-box), which are likely to affect binding capacity of the polymerase III transcriptional machinery. However, none of the mutations affected *AP-A trans* inactivation capacity, indicating that the presence of the tRNA gene, but not its transcription or binding of the transcriptional machinery, is required for *trans* inactivation. NA, not applicable.

understand the architecture of the amoebae nucleus are necessary to finally prove this hypothesis.

A tgm-like mechanism. The fact that *AP-A trans* inactivation depends on the presence of tRNA gene sequences suggests a tgm-related mechanism, which has been recognized so far exclusively in *S. cerevisiae*. For *S. cerevisiae*, Pol III transcription of tRNAs has been described as a central step of tgm required for their spatial clustering (18). However, in contrast to the case in *S. cerevisiae*, tgm in *E. histolytica* does not appear to depend on transcription or binding of the polymerase III machinery, as none of the mutations introduced into the two regulatory elements of the amoeba tyrosine tRNA gene did abolish the *trans* inactivation capacity of the transfected plasmid. If transcription or Pol III binding of tRNA genes were the cause for silencing, any mutations that cause loss of transcription or Pol III binding in one gene would yield the same effect as the deletion of that gene. However, tyrosine tRNA gene mutations did not even moderately impair *trans* inactivation, while its deletion completely abolished any silencing capacity. We suggest that binding of the transcriptional machinery (or transcription) may not be required for spatial clustering in *E. histolytica*, because amoeba tRNA genes are already "clustered" due to the fact that they are arranged in long tandem-repeat arrays (9).

In the search for a putative silencer element, a PD1 homologue was identified in the *AP-A* upstream region (42). However, deletion or mutation of this element did not interfere with the *trans* inactivation capacity of psAP1 (Fig. 6). As no other sequence similarities to known silencer elements have been identified, silencer elements are either not present or not well conserved between *E. histolytica* and other eukaryotes. The latter explanation seems more likely, as the molecular mechanisms of heterochromatin formation and gene silencing appear to be only weakly conserved in *E. histolytica*. For many of the proteins known to be involved in heterochromatin formation or silencing, there is no evidence of their existence in the genome of *E. histolytica* (data not shown).

There remain questions about the specific properties of HM-1:IMSS (2411) that enable transcriptional silencing in this particular strain but not in others. So far, various attempts to identify strain-specific differences have been made. These include PCR-based strain typing and sequencing of the AP-A locus and the tRNA array as well as microarray analyses using panels of selected amoeba genes (4, 22, 38) or a panel comprising protein-coding genes of the entire *E. histolytica* genome (D. Mirelman, personal communication). However, none of these approaches resolved the problem, suggesting that an

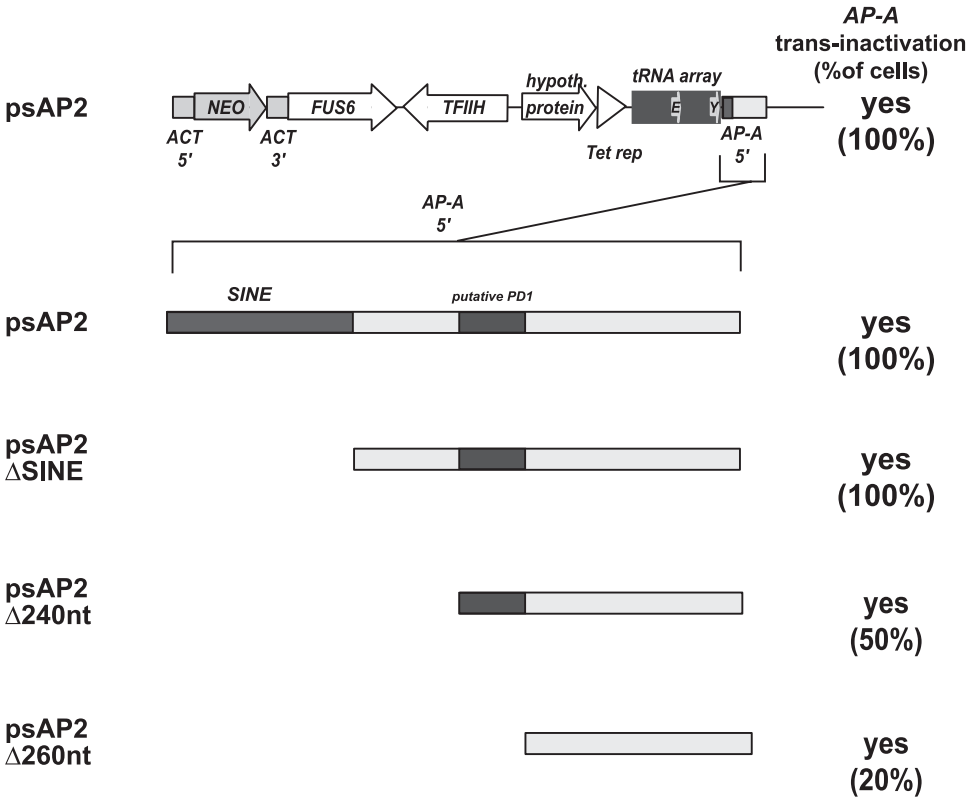


FIG. 6. SINE and a putative PD1 element are dispensable for *AP-A trans* inactivation. The silencing plasmid psAP2 containing largely the same sequence elements as psAP1 but lacking the *AP-A* coding and 3′ flanking sequences was used to analyze the requirement for SINE and the putative PD1 element for *AP-A trans* inactivation. Various 5′ deletion mutant forms of the *AP-A* upstream region were generated and substituted for the *AP-A* 5′ region in psAP2 as indicated. Even the minimal fragment tested (psAP2Δ260nt) comprising 213 bp of the *AP-A* upstream region but lacking SINE as well as PD1 mediated *trans* inactivation. Note that in contrast to the longer fragments, which silenced *AP-A* in 100% of transfected amoebae, deletion plasmids psAP2Δ240nt and psAP2Δ260nt induced *trans* inactivation efficiency in only 50% and 20% of transfected cells, respectively. *NEO*, neomycin resistance gene.

epigenetic phenomenon or a mutation may affect any yet unknown gene involved in silencing.

Future comparative genome sequencing may help to decipher the molecular differences between the strains, thus providing further insights into the mechanism of gene silencing in *E. histolytica*.

Another important result of this study is that AP-A expression can be restored in *AP-A*-silenced amoeba by episomal transfections using the *AP-A* open reading frame under the control of a heterologous amoeba promoter including an intron with enhancer properties. This *AP-A* add-back mutant led to an abundance of AP-A similar to that of nonsilenced wild-type cells. This is in contrast to a study in which reconstitution of AP-A expression could not be achieved by episomal transfection (5), likely due to differences in the transfection vectors. Irrespectively, reexpression of genes by episomal transfection is a crucial advancement toward more sophisticated investigations of amoeba protein function, cell biology, and pathogenicity, as targeted silencing of genes of interest currently can be achieved only in amoebae in which *AP-A* is additionally silenced (4, 6, 22). However, AP-A-deficient cells are highly impaired in pathogenicity and therefore not suitable for the investigation of other pathogenicity factors.

ACKNOWLEDGMENTS

We thank David Mirelman for helpful discussions and for providing the psAP1 plasmid and HM-1:IMSS (2411) cells. We also thank Matthias Leippe for anti-AP-A and anti-AP-B antisera, and we thank Susann Ofori, Fabian Peters, Nicola Porter, and Heidrun von Thien for skillful technical assistance.

REFERENCES

1. **Ali, I. K., M. Zaki, and C. G. Clark.** 2005. Use of PCR amplification of tRNA gene-linked short tandem repeats for genotyping *Entamoeba histolytica*. J. Clin. Microbiol. **43:**5842–5847.
2. **Anbar, M., R. Bracha, Y. Nuchamowitz, Y. Li, A. Florentin, and D. Mirelman.** 2005. Involvement of a short interspersed element in epigenetic transcriptional silencing of the amoebapore gene in *Entamoeba histolytica*. Eukaryot. Cell **4:**1775–1784.
3. **Andrulis, E. D., A. M. Neiman, D. C. Zappulla, and R. Sternglanz.** 1998. Perinuclear localization of chromatin facilitates transcriptional silencing. Nature **394:**592–595.
4. **Bracha, R., Y. Nuchamowitz, M. Anbar, and D. Mirelman.** 2006. Transcriptional silencing of multiple genes in trophozoites of *Entamoeba histolytica*. PLoS Pathog. **2:**e48.
5. **Bracha, R., Y. Nuchamowitz, and D. Mirelman.** 2003. Transcriptional silencing of an amoebapore gene in *Entamoeba histolytica*: molecular analysis and effect on pathogenicity. Eukaryot. Cell **2:**295–305.
6. **Bracha, R., Y. Nuchamowitz, N. Wender, and D. Mirelman.** 2007. Transcriptional gene silencing reveals two distinct groups of *Entamoeba histolytica* Gal/GalNAc-lectin light subunits. Eukaryot. Cell **6:**1758–1765.
7. **Brown, K. E., S. S. Guest, S. T. Smale, K. Hahm, M. Merkenschlager, and A. G. Fisher.** 1997. Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin. Cell **91:**845–854.

8. **Buck, S. W., J. J. Sandmeier, and J. S. Smith.** 2002. RNA polymerase I propagates unidirectional spreading of rDNA silent chromatin. Cell **111:** 1003–1014.
9. **Clark, C. G., I. K. Ali, M. Zaki, B. J. Loftus, and N. Hall.** 2006. Unique organisation of tRNA genes in *Entamoeba histolytica*. Mol. Biochem. Parasitol. **146:**24–29.
10. **Csink, A. K., and S. Henikoff.** 1996. Genetic modification of heterochromatic association and nuclear organization in Drosophila. Nature **381:**529–531.
11. **Diamond, L. S., D. R. Harlow, and C. C. Cunnick.** 1978. A new medium for the axenic cultivation of *Entamoeba histolytica* and other Entamoeba. Trans. R. Soc. Trop. Med. Hyg. **72:**431–432.
12. **Duncan, I. W.** 2002. Transvection effects in Drosophila. Annu. Rev. Genet. **36:**521–556.
13. **Ehrenhofer-Murray, A. E., M. Gossen, D. T. Pak, M. R. Botchan, and J. Rine.** 1995. Separation of origin recognition complex functions by cross-species complementation. Science **270:**1671–1674.
14. **Fisher, A. G., and M. Merkenschlager.** 2002. Gene silencing, cell fate and nuclear organisation. Curr. Opin. Genet. Dev. **12:**193–197.
15. **Funabiki, H., I. Hagan, S. Uzawa, and M. Yanagida.** 1993. Cell cycle-dependent specific positioning and clustering of centromeres and telomeres in fission yeast. J. Cell Biol. **121:**961–976.
16. **Grünweller, A., and A. E. Ehrenhofer-Murray.** 2002. A novel yeast silencer. the 2mu origin of Saccharomyces cerevisiae has HST3-, MIG1- and SIR-dependent silencing activity. Genetics **162:**59–71.
17. **Haeusler, R. A., and D. R. Engelke.** 2006. Spatial organization of transcription by RNA polymerase III. Nucleic Acids Res. **34:**4826–4836.
18. **Haeusler, R. A., M. Pratt-Hyatt, P. D. Good, T. A. Gipson, and D. R. Engelke.** 2008. Clustering of yeast tRNA genes is mediated by specific association of condensin with tRNA gene transcription complexes. Genes Dev. **22:**2204–2214.
19. **Hamann, L., R. Nickel, and E. Tannich.** 1995. Transfection and continuous expression of heterologous genes in the protozoan parasite *Entamoeba histolytica*. Proc. Natl. Acad. Sci. U. S. A. **92:**8975–8979.
20. **Huber, M., B. Koller, C. Gitler, D. Mirelman, M. Revel, S. Rozenblatt, and L. Garfinkel.** 1989. *Entamoeba histolytica* ribosomal RNA genes are carried on palindromic circular DNA molecules. Mol. Biochem. Parasitol. **32:**285–296.
21. **Hull, M. W., J. Erickson, M. Johnston, and D. R. Engelke.** 1994. tRNA genes as transcriptional repressor elements. Mol. Cell. Biol. **14:**1266–1277.
22. **Irmer, H., M. Tillack, L. Biller, G. Handal, M. Leippe, T. Roeder, E. Tannich, and I. Bruchhaus.** 2009. Major cysteine peptidases of *Entamoeba histolytica* are required for aggregation and digestion of erythrocytes but are dispensable for phagocytosis and cytopathogenicity. Mol. Microbiol. **72:**658–667.
23. **Jhingan, G. D., S. K. Panigrahi, A. Bhattacharya, and S. Bhattacharya.** 2009. The nucleolus in *Entamoeba histolytica* and *Entamoeba invadens* is located at the nuclear periphery. Mol. Biochem. Parasitol. **167:**72–80.
24. **Kendall, A., M. W. Hull, E. Bertrand, P. D. Good, R. H. Singer, and D. R. Engelke.** 2000. A CBF5 mutation that disrupts nucleolar localization of early tRNA biosynthesis in yeast also suppresses tRNA gene-mediated transcriptional silencing. Proc. Natl. Acad. Sci. U. S. A. **97:**13108–13113.
25. **Leippe, M., S. Ebel, O. L. Schoenberger, R. D. Horstmann, and H. J. Muller-Eberhard.** 1991. Pore-forming peptide of pathogenic *Entamoeba histolytica*. Proc. Natl. Acad. Sci. U. S. A. **88:**7659–7663.
26. **Liu, H., J. Huang, J. Wang, S. Jiang, A. S. Bailey, D. C. Goldman, M. Welcker, V. Bedell, M. L. Slovak, B. Clurman, et al.** 2008. Transvection mediated by the translocated cyclin D1 locus in mantle cell lymphoma. J. Exp. Med. **205:**1843–1858.
27. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods **25:**402–408.
28. **Loftus, B., I. Anderson, R. Davies, U. C. Alsmark, J. Samuelson, P. Amedeo, P. Roncaglia, M. Berriman, R. P. Hirt, B. J. Mann, et al.** 2005. The genome of the protist parasite *Entamoeba histolytica*. Nature **433:**865–868.
29. **Lohia, A., N. Haider, and B. B. Biswas.** 1990. Characterisation of a repetitive DNA family from *Entamoeba histolytica* containing *Saccharomyces cerevisiae* ARS consensus sequences. Gene **96:**197–203.
30. **Loo, S., and J. Rine.** 1995. Silencing and heritable domains of gene expression. Annu. Rev. Cell Dev. Biol. **11:**519–548.
31. **Marcand, S., S. W. Buck, P. Moretti, E. Gilson, and D. Shore.** 1996. Silencing of genes at nontelomeric sites in yeast is controlled by sequestration of silencing factors at telomeres by Rap 1 protein. Genes Dev. **10:**1297–1309.
32. **Mirelman, D., M. Anbar, and R. Bracha.** 2008. Trophozoites of *Entamoeba histolytica* epigenetically silenced in several genes are virulence-attenuated. Parasite **15:**266–274.
33. **Rassoulzadegan, M., M. Magliano, and F. Cuzin.** 2002. Transvection effects involving DNA methylation during meiosis in the mouse. EMBO J. **21:**440–450.
34. **Sage, B. T., J. L. Jones, A. L. Holmes, M. D. Wu, and A. K. Csink.** 2005. Sequence elements in cis influence heterochromatic silencing in trans. Mol. Cell. Biol. **25:**377–388.
35. **Sass, G. L., and S. Henikoff.** 1999. Pairing-dependent mislocalization of a Drosophila brown gene reporter to a heterochromatic environment. Genetics **152:**595–604.
36. **Schägger, H., H. Aquila, and G. Von Jagow.** 1988. Coomassie blue-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for direct visualization of polypeptides during electrophoresis. Anal. Biochem. **173:**201–205.
37. **Simms, T. A., E. C. Miller, N. P. Buisson, N. Jambunathan, and D. Donze.** 2004. The *Saccharomyces cerevisiae* TRT2 tRNAThr gene upstream of STE6 is a barrier to repression in MATalpha cells and exerts a potential tRNA position effect in MATa cells. Nucleic Acids Res. **32:**5206–5213.
38. **Tillack, M., L. Biller, H. Irmer, M. Freitas, M. A. Gomes, E. Tannich, and I. Bruchhaus.** 2007. The *Entamoeba histolytica* genome: primary structure and expression of proteolytic enzymes. BMC Genomics **8:**170.
39. **Towbin, B. D., P. Meister, and S. M. Gasser.** 2009. The nuclear envelope—a scaffold for silencing? Curr. Opin. Genet. Dev. **19:**180–186.
40. **van Driel, R., P. F. Fransz, and P. J. Verschure.** 2003. The eukaryotic genome: a system regulated at different hierarchical levels. J. Cell Sci. **116:** 4067–4075.
41. **Wang, L., R. A. Haeusler, P. D. Good, M. Thompson, S. Nagar, and D. R. Engelke.** 2005. Silencing near tRNA genes requires nucleolar localization. J. Biol. Chem. **280:**8637–8639.
42. **Weissman, J. D., and D. S. Singer.** 1991. A complex regulatory DNA element associated with a major histocompatibility complex class I gene consists of both a silencer and an enhancer. Mol. Cell. Biol. **11:**4217–4227.
43. **Willhoeft, U., and E. Tannich.** 2000. Fluorescence microscopy and fluorescence in situ hybridization of *Entamoeba histolytica* nuclei to analyse mitosis and the localization of repetitive DNA. Mol. Biochem. Parasitol. **105:**291–296.