Retrotransposons are ancient mobile elements that amplify in eukaryotic cells via reverse transcription of RNA intermediates (6). Because genomic integration of retrotransposon-derived cDNA is a default mechanism in the amplification of these elements, their activity causes a constant threat of insertion mutagenesis and genome instability in host genomes. This threat may be particularly important if the host cell has a haploid and compact genome, such as that of the social amoeba Dictyostelium discoideum, which some 67% of nuclear DNA codes for proteins (7). D. discoideum mobile elements display two different regional integration preferences (reviewed in reference 28). The first group of mobile elements shows a strong bias toward inserting into preexisting copies of the same or similar elements, thereby forming large clusters in certain chromosomal regions that rarely contain protein coding capacity. A second group of retrotransposons has developed mechanisms to actively target sites in the close vicinity of tRNA genes as integration sites by making contact between the 5′ end of the TRE5-A element (22). A C-module-binding factor (CbfA) was purified and characterized based on its binding to the C module of TRE5-A in electrophoretic mobilization assays (9, 10, 24, 27, 29). The CbfA protein spans 1,000 amino acids and contains a “carboxy-terminal Jumonji domain” (JmjC), two zinc finger-like motifs of unknown function, an asparagine-rich domain, and a distinct carboxy-terminal JmjC domain (referred to here as CbfA-CTD). JmjC domains are thought to catalyze the oxidative demethylation of histone tails in chromatin (reviewed in references 1, 11, and 26), thereby contributing to the epigenetic control of gene transcription. It is currently unknown whether CbfA has chromatin-remodeling activity.

DNA microarray analyses have indicated that CbfA regulates at least 160 genes during the growth phase of D. discoi-
deum (14). CbfA is also essential to initiating the multicellular life cycle of D. discoideum cells, probably because CbfA mutants are unable to induce the adenyllyl cyclase that generates the cyclic AMP required for intercellular signaling during aggregation (27). Interestingly, DNA microarray analyses have also revealed a gene regulatory function of CbfA-CTD that does not require the remainder of the CbfA protein. In fact, some 50% of CbfA-dependent genes are regulated exclusively by CbfA-CTD (14).

Here we show that the retrotransposition of TRE5-A in D. discoideum cells depends on a functional CbfA protein. Whereas CbfA-CTD is sufficient to maintain high steady-state levels of both (+) RNA and (−) RNA of TRE5-A, it does not support productive retrotransposition of these TRE5-A transcripts in the absence of the full-length CbfA protein. This finding suggests different roles for CbfA protein domains in the regulation of the TRE5-A retrotransposition frequency in D. discoideum cells.

MATERIALS AND METHODS

In vivo retrotransposition assay (TRE trap assay). JH.D[ura−] cells were derived from strain JH.LD (29) by prolonged selection of cells in the presence of 100 μg/ml 5-fluoroorotic acid (5-FOA) and 20 μg/ml uracil. DH1[ura−] (3) and JH.D[ura−] cells were cultured either in HL5 medium or in FM medium supplemented with 20 μg/ml uracil. The TRE trap assay was performed as described by Siol et al. (23). Briefly, DH1[ura−] or JH.D[ura−] cells were transformed with a plasmid carrying a D. discoideum pyr5-6 gene whose reading frame was disrupted by an artificial intron. A D. discoideum Val®AC RNA gene was inserted into the intron as bait to attract TRE5-A integrations. These constructs are referred to as TRE5-A genes. TRE®A carrying plasmids were transformed into DH1[ura−] or JH.D[ura−] cells, and transformants were selected in FM medium in the absence of uracil. DH1[ura−]/TRE®A or JH.D[ura−]/TRE®A cells were cultured further in HL5 medium and were supertransformed either with plasmid pDXA-cbA1, expressing nearly-full-length CbfA2-998 (27), or with pDXA-CTD, expressing the carboxy-terminal domain of CbfA (CbfA-CTD; CbfA724-998) (14). Stable transformants were obtained by selection in HL5 medium containing 4 μg/ml G418. To select for de novo retrotransposition events, 107 cells of the respective transformants were cultured in FM medium in the presence of 250 μg/ml 5-FOA and 30 μg/ml uracil, as described previously (2, 23). TRE®A/TRE®A− clones from five different plates were counted, and mean values ± standard deviations (SD) were presented. Each experiment was repeated at least twice.

Luciferase reporter assay. An A module of TRE5-A.1 was amplified by PCR from plasmid pB3 (18) and inserted into pGEM-T (Promega). A firefly luciferase gene, including the cotB terminator, was isolated by HindIII/SpeI fragment from plasmid pVTL-AL (12) and then ligated into the intron as bait to attract TRE5-A integrations. These constructs are referred to as TRE trap genes. TREtrap-carrying plasmids were transformed into D. discoideum vector-specific primers at both ends of the cloned insert.

RESULTS

Accumulation of TRE5-A transcripts in D. discoideum cells requires CbfA. Although CbfA is required to regulate the expression of a multitude of genes in the D. discoideum genome (14), its original isolation as a C-module-binding factor suggested an additional role for the factor in the regulation of TRE5-A transcript levels and/or retrotransposition activity. To evaluate whether CbfA has an influence on the steady-state transcript levels of TRE5-A, we conducted quantitative RT-PCR on poly(dT)-primed cDNA preparations. Considering that retrotransposon activity is a source of genome instability, we expected that the function of CbfA might be to suppress the accumulation of high levels of TRE5-A transcripts, the prerequisite for retrotransposition. To our surprise, we observed that the depletion of CbfA from D. discoideum cells in the mutant strain JH.D did not lead to stabilization, but rather to an increase of TRE5-A transcripts. A reduction in TRE5-A expression was determined, by a factor of 5.3 ± 1.5 (n = 7), compared with that of the parent strain AX2.

To evaluate whether the accumulation of both (+) RNA and (−) RNA TRE5-A transcripts was affected in CbfA-depleted cells, we prepared Northern blots of total RNA from D. discoideum cells and used strand-specifically radiolabeled DNA probes to visualize (+) RNA and (−) RNA. To increase the sensitivity of the assay, we treated cells in parallel cultures with the respiratory chain blocker antimycin A, which is known to increase the amount of TRE5-A transcripts in growing D. discoideum cells (22). As shown in Fig. 1, the levels of both (+) RNA and (−) RNA were drastically reduced in the absence of a functional CbfA protein.

To determine if the observed loss of TRE5-A transcripts in the CbfA mutant was in fact related to the depletion of CbfA, we performed complementation studies using two plasmid-borne CbfA variants. The first was a nearly-full-length CbfA2-998 protein expressed from the strong actI5 promoter.
The CbfA<sub>724-998</sub> protein proved to be functional in restoring the aggregation phenotype of JH.D cells (27). The second was the carboxy-terminal domain of CbfA (CbfA<sub>724-998</sub>; CbfA-CTD), also expressed from the act15 promoter, which is known to act as an independent gene regulatory entity for about half of all CbfA-regulated genes (14). When either full-length CbfA or CbfA-CTD was produced in the JH.D background, complete reversion of both TRE5-A<sup>H11001</sup> and TRE5-A<sup>H11002</sup> RNA expression was observed (Fig. 2).

A possible explanation for the simultaneous loss of TRE5-A<sup>H11001</sup> and TRE5-A<sup>H11002</sup> RNA in the CbfA mutant could be direct positive regulation of the two responsible TRE5-A promoters, the A module and the C module, by CbfA. To test this possibility, we inserted the A module and the C module upstream of a luciferase reporter gene in a <i>D. discoideum</i> expression vector. AX2 and JH.D cells were transformed with these vectors, and luciferase reporter gene activity was determined. Both promoters mediated expression of luciferase, with reporter activity above the background, but we did not observe significant differences in A-module or C-module promoter activity in the CbfA-depleted mutant JH.D cells in comparison to AX2 cells (Fig. 3).

CbfA increases TRE5-A retrotransposition activity in <i>D. discoideum</i> cells. The results described above indicate that CbfA is a host factor for TRE5-A expression in growing <i>D. discoideum</i> cells. We wanted to determine whether CbfA increased not only the steady-state levels of TRE5-A-derived transcripts but also the retrotransposition frequency of the element. First, we measured the frequency of <i>de novo</i> TRE5-A retrotransposition events in the CbfA-depleted mutant strain JH.D, using the previously established “TRE trap” assay. This assay reliably reflects the natural retrotransposition activity of the TRE5-A population in the <i>D. discoideum</i> genome (2, 23). Briefly, the TRE trap assay consists of a cloned <i>D. discoideum</i> UMP synthase gene (<i>pyr5</i>-6) that contains an artificial intron (Fig. 4). Into this intron, we placed a tRNA gene that served as a target for integration of TRE5-A elements, thus disrupting the entire <i>pyr5</i>-6 gene. When such a TRE<sup>trap</sup> gene was transformed into the ura<sup>H11002</sup><i>D. discoideum</i> strain DH1, the TRE<sup>trap</sup> plasmid readily complemented the ura<sup>H11002</sup> phenotype and converted the cells to a ura<sup>H11001</sup> phenotype. Such cells, however, were
sensitive to the cytostatic drug 5-FOA and were killed unless they acquired mutations in the TRE\textsuperscript{trap} gene. Under selection with 5-FOA and uracil, resistant ura\textsuperscript{+/H11002} clones appeared as a consequence of the disruption of the TRE\textsuperscript{trap} gene by the de novo integration of endogenous active TRE5-A elements (Fig. 5). As a negative control, we used a TRE\textsuperscript{trap} plasmid that lacked the bait tRNA gene. Under these conditions, the TRE\textsuperscript{trap} bait did not attract TRE5-A retrotransposons; only a few clones were obtained (Fig. 5), and these probably arose from spontaneous mutations of the TRE\textsuperscript{trap} gene rather than targeted integration of TRE5-A elements (data not shown; discussed in reference 2).

We developed ura\textsuperscript{−} mutants in CbfA-depleted JH.D cells by prolonged selection of cells in the presence of 5-FOA and uracil. For further experiments, we chose a JH.D[ura\textsuperscript{−}]/H11002 mutant that had a complete chromosomal deletion of the pyr5-6 coding region, as determined by PCR analysis (data not shown). We transformed TRE\textsuperscript{trap} plasmids into the JH.D[ura\textsuperscript{−}] cells and subjected JH.D[ura\textsuperscript{−}/TRE\textsuperscript{trap}] transformants to 5-FOA selection. As shown in Fig. 5, CbfA depletion of D. discoideum cells resulted in a ~90% reduction in TRE5-A retrotransposition activity. We noticed a reproducible increase in background ura\textsuperscript{−} clones obtained from JH.D[ura\textsuperscript{−}/TRE\textsuperscript{trap}] transformants after 5-FOA selection compared to those obtained from DH1[ura\textsuperscript{−}/TRE\textsuperscript{trap}] cells in the absence of a target tRNA gene (Fig. 5). This increase may reflect a generally higher mutation rate in CbfA-depleted cells, but this was not analyzed further.

Although these data were promising, they were challenged by the different strain histories of the cells used in this experiment: whereas the ura\textsuperscript{−} mutant DH1 (the wild-type strain in this experiment) was derived from the axenic D. discoideum strain AX3, our CbfA mutant strain (JH.D) was derived from AX2. Although AX3 and AX2 have the same parent strain, NC4, the different laboratory histories of both strains may have corrupted TRE5-A retrotransposition activity in AX2 cells, thus mimicking a CbfA effect on TRE5-A retrotransposition in JH.D cells. We therefore decided to perform complementation studies in JH.D[ura\textsuperscript{−}] cells expressing plasmid-borne CbfA to rescue the aberrant TRE5-A retrotransposition activities. In two parallel series of transformations, TRE\textsuperscript{trap} plasmids that either contained or lacked a target tRNA gene were introduced into JH.D[ura\textsuperscript{−}] cells. The resulting transformants were supertransformed with plasmids that conferred resistance to G418 and supported the expression of CbfA\textsuperscript{2-998} or CbfA-CTD. First, stable transformants were screened for comparable expression of the plasmid-borne CbfA variants (data not shown). Next, we measured the relative expression levels of TRE5-A in the transformants by quantitative RT-PCR (Fig. 6A). As expected from our data described above, we found that the expression of CbfA\textsuperscript{2-998} or CbfA-CTD increased the expression of TRE5-A in cells carrying the TRE\textsuperscript{trap} gene. Finally, we measured the retrotransposition activity of the TRE5-A population in the TRE trap assay. The natural mutation rate observed for the TRE\textsuperscript{trap} gene was approximately 45 clones per 10\textsuperscript{7} cells (Fig. 6B). This rate was not increased by the ectopic expression of CbfA\textsuperscript{2-998} or CbfA-CTD (Fig. 6B). In contrast, when a target tRNA gene was included in the TRE\textsuperscript{trap} gene, the mutation rate at the TRE\textsuperscript{trap} gene increased approximately 4-fold in the presence of full-length CbfA, to roughly the wild-type levels observed in DH1 cells. This observation indicated full rescue of retrotransposition activity of the TRE5-A population. Unexpectedly, we found that expression of CbfA-CTD, although fully restoring aberrant TRE5-A expression, did not rescue the retrotransposition deficiency of the CbfA mutant. This result was observed in three independent experiments, and the reason for it remains obscure.

**DISCUSSION**

CbfA is a host factor that supports TRE5-A expression. In this report, we describe experiments demonstrating that CbfA is a host factor for TRE5-A expression in growing D. discoideum cells. In contrast to our prediction, we found that CbfA does not act as a component of the cellular defense machinery that is expected to limit the expression and subsequent ampli-
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TRE5-A. In some organisms, for example, *Drosophila*, RNA interference (RNAi) is strictly ATP dependent (30), while in other organisms, such as humans (as demonstrated with human cells), RNAi seems to function in an ATP-independent manner (31). For *D. discoideum*, it is postulated that all known PTGS effects are mediated by RNA-dependent RNA polymerases (20), meaning that PTGS requires highly energy-consuming RNA-amplifying steps to work. Thus, assuming that antimycin A limits the energy metabolism in *D. discoideum* cells by blocking respiratory chain function, one could argue that antimycin affects TRE5-A transcript levels by blocking PTGS mechanisms. The role of CbfA in the stabilization of (+) RNA and (−) RNA of TRE5-A remains elusive. We speculate that CbfA regulates the expression of a gene involved in PTGS. This question will be addressed in future studies.

**CbfA is essential for TRE5-A retrotransposition.** Although CbfA-CTD is required and sufficient to maintain high TRE5-A transcript levels in *D. discoideum* cells, it was not able to restore the diminished retrotransposition of TRE5-A in the CbfA mutant. This result suggested a role for CbfA in the retrotransposition process in addition to the regulation of TRE5-A expression. Is CbfA a component of the TRE5-A preintegration complex? All available data indicate that TRE5-A is a canonical non-LTR retrotransposon that integrates via target-primed reverse transcription (TPRT), which means that no free double-stranded DNA intermediates of TRE5-A are supposed to occur in the cell to which CbfA may bind through its DNA-binding capacity. It also seems improbable that CbfA binds to TRE5-A-derived RNA because CbfA does not contain predictable RNA-binding domains or possess other enzymatic functions presumably required for the reverse transcription and integration processes. Although productive integration of mobilized retrotransposons may rely on the activity of host factors such as DNA repair enzymes, the reverse transcription and integration of such elements are likely to be mediated exclusively by TRE5-A ORF1/ORF2 functions. We concluded that it is unlikely that host-encoded CbfA has a direct function in the TPRT process.

Instead, the retrotransposition frequency of TRE5-A may be affected indirectly by CbfA if a component of the cellular DNA repair machinery exists that is expressed in a CbfA-dependent manner and cannot be controlled by CbfA-CTD alone. Support for this assumption may be deduced from the observation that we reproducibly obtained more ura− mutants in the TRE trap assay with JH.D cells than in that with DH1 wild-type cells in the absence of a tRNA gene (columns 1 and 3 in Fig. 5), a sign of an increased general mutation rate in the mutant. However, we assume that nonproductive attempts by TRE5-A to integrate upstream of the bait tRNA gene in the TRE trap assay may have caused substantial genome instability at this locus due to insufficient DNA repair and, in turn, should have generated excess ura− mutants. However, excess ura− mutants were not observed for the CbfA mutant (column 4 in Fig. 5). On the contrary, we found that retrotransposon-induced mutagenesis of the TRE5-A gene was almost completely lost in the absence of CbfA.

One could imagine that the normal cellular function of CbfA as a putative chromatin-remodeling enzyme may provide a chromatin architecture in the vicinity of tRNA genes that sup-

![FIG. 6.](image-url) Complementation of TRE5-A retrotransposition in the CbfA mutant. (A) TRE5-A expression was quantified in JH.D/TRE<sup>trap</sup>/ura−] transformants by real-time RT-PCR. Expression of TRE5-A in AX2 cells versus untransformed JH.D/ura−] cells served as a control (white bar, column 1). JH.D/ura−] cells were transformed with TRE<sup>trap</sup> plasmids without a tRNA gene as an integration target (gray bars, columns 2 to 4) or with a *D. discoideum* Val<sup>DAC</sup> tRNA gene as bait to attract mobile TREs (black bars, columns 5 to 7). Resulting JH.D/TRE<sup>trap</sup>/ura−] cells were then supertransformed with empty expression vector (columns 2 and 5) or with plasmids that allowed for the expression of either CbfA-CTD (columns 3 and 6) or full-length CbfA (columns 4 and 7) in the JH.D background. All expression data were obtained from 4 to 8 independent transformants and are given relative to untransformed JH.D/ura−] cells, meaning that values of >1 indicate more TRE5-A expression in the transformants or AX2 cells than in untransformed JH.D/ura−] cells. (B) JH.D/TRE<sup>trap</sup>/ura−] cells were subjected to selection in 5-FOA and uracil. Numbers of 5-FOA-resistant clones were calculated for 5 petri dishes and are given as means ± SD. Columns are the same as in panel A. The experiment was repeated twice with similar results.
ports the integration process. This is speculative as long as the putative functions of the JmjC and zinc finger domains of CbfA remain elusive.

In conclusion, CbfA is a host-encoded factor that strongly supports TRE5-A amplification in modern *D. discoideum* strains by controlling the absolute amount of transcript that is available for translation of TRE5-A proteins, reverse transcription of TRE5-A (+) RNA, and subsequent integration into chromosomal loci. CbfA has two distinct, indirect functions that can be assigned to different parts of the protein: maintenance of high steady-state levels of TRES-A transcripts requires only the carboxy-terminal domain of CbfA, whereas the remaining parts of the protein, perhaps utilizing chromatin-remodeling activity of CbfA, are required to pass through a complete retrotransposition cycle.

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