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Epigenetic Silencing of a Foreign Gene in Nuclear Transformants of Chlamydomonas

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The unstable expression of introduced genes poses a serious problem for the application of transgenic technology in plants. In transformants of the unicellular green alga *Chlamydomonas reinhardtii*, expression of a eubacterial *aadA* gene, conferring spectinomycin resistance, is transcriptionally suppressed by a reversible epigenetic mechanism(s). Variations in the size and frequency of colonies surviving on different concentrations of spectinomycin as well as the levels of transcriptional activity of the introduced transgene(s) suggest the existence of intermediate expression states in genetically identical cells. Gene silencing does not correlate with methylation of the integrated DNA and does not involve large alterations in its chromatin structure, as revealed by digestion with restriction endonucleases and DNase I. Transgene repression is enhanced by lower temperatures, similar to position effect variegation in Drosophila. By analogy to epigenetic phenomena in several eukaryotes, our results suggest a possible role for (hetero)chromatic chromosomal domains in transcriptional inactivation.

INTRODUCTION

The unicellular green alga *Chlamydomonas reinhardtii* has long been used to study diverse biological processes such as photosynthesis, organelle biogenesis, and flagellar assembly and function (Harris, 1989). In addition to its advantages for genetic analysis, Chlamydomonas is at present the only photosynthetic organism in which the nuclear, chloroplast, and mitochondrial genomes can be stably transformed (Boynton et al., 1988; Kindle et al., 1989; Kindle, 1990; Randolph-Anderson et al., 1993; Cerutti et al., 1997). The integration of transforming DNA into the Chlamydomonas nuclear genome occurs predominantly by nonhomologous recombination, and the transgenes are stably transmitted as Mendelian traits (Kindle et al., 1989; Kindle, 1990; Tam and Lefebvre, 1993; Cerutti et al., 1997). However, full exploitation of Chlamydomonas as a model system has been hindered by difficulties encountered in expressing foreign sequences integrated into the nuclear genome (Blankenship and Kindle, 1992; Stevens et al., 1996; Cerutti et al., 1997). Even intact or chimeric Chlamydomonas genes reintroduced into the nuclear genome by transformation are often expressed at reduced and quite variable levels when compared with the endogenous genes (Blankenship and Kindle, 1992; Kozminski et al., 1993; Quinn and Merchant, 1995). The reasons for the failure to express heterologous transgenes and for the variable expression of homologous transgenes in the Chlamydomonas nuclear genome are not known, although we have recently suggested that gene silencing may play a role (Cerutti et al., 1997). Similarly, the unstable expression of introduced genes in higher plants, often influenced in unpredictable ways by environmental and/or developmental factors, constitutes a serious problem for the use of transgenic technology for both basic and applied purposes (Matzke and Matzke, 1995; Meyer and Saedler, 1996).

In plants, animals, and fungi, expression of introduced genes and/or their endogenous homologs sometimes becomes reversibly suppressed, as manifested by reduced accumulation of the corresponding mRNAs (Rossignol and Faugeron, 1994; Hendrich and Willard, 1995; Henikoff, 1995; Matzke and Matzke, 1995; Singer and Selker, 1995; Weiler and Wakimoto, 1995; Meyer and Saedler, 1996). This gene inactivation results from a variety of epigenetic mechanisms that underlie, among others, the following phenomena: (1) homology-dependent gene silencing and paramutation in higher plants (Brink, 1973; Matzke and Matzke, 1995; Patterson and Chandler, 1995; Meyer and Saedler, 1996); (2) repeat-induced point mutation in *Neurospora crassa* (Singer and Selker, 1995); (3) methylation induced premeiotically in *Ascobolus immersus* and *Coprinus cinereus* (Rossignol and Faugeron, 1994); (4) position effect variegation in Drosophila (Karpen, 1994; Henikoff, 1995; Weiler and Wakimoto, 1995;

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Elgin, 1996); (5) chromatin-mediated silencing in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Allshire et al., 1994; Aparicio and Gottschling, 1994; Karpen, 1994; Loo and Rine, 1994); and (6) variegated transgene expression, X chromosome inactivation, and genomic imprinting in mammals (Hendrich and Willard, 1995; Festenstein et al., 1996; Milot et al., 1996).

To our knowledge, epigenetic gene silencing has not been demonstrated in algae. However, in higher plants as well as in filamentous fungi (Rossignol and Faugeron, 1994; Singer and Selker, 1995; Cogoni et al., 1996), gene inactivation occurs most often when there are multiple copies of a particular sequence in the genome. Homology-dependent gene silencing in plants seems to result from several distinct mechanisms that are postulated to involve either direct or protein-mediated DNA–DNA, DNA–RNA, or RNA–RNA interactions (Matzke and Matzke, 1995; Meyer and Saedler, 1996). In some cases, this gene inactivation involves transcriptional repression, shows at least partial meiotic heritability, and has been correlated with cytosine methylation, particularly of the promoter regions (Meyer et al., 1993; Neuhuber et al., 1994; Park et al., 1996). In a few examples, transcriptional inactivation also seems to be accompanied by a condensed chromatin structure (Ten Lohuis et al., 1995; Ye and Signer, 1996). Transcriptional silencing of some transgenes is strongly reminiscent of paramutation, particularly at the maize *b* locus (encoding a transcriptional activator of the anthocyanin biosynthetic pathway) (Matzke and Matzke, 1995; Patterson and Chandler, 1995; Patterson et al., 1995; Meyer and Saedler, 1996).

In other cases of homology-dependent gene silencing in plants, particularly those involving duplicated coding sequences, gene repression appears to take place post-transcriptionally, resulting in increased turnover of homologous RNAs (Dehio and Schell, 1994; de Carvalho Niebel et al., 1995; Elmayan and Vaucheret, 1996; Goodwin et al., 1996; Kunz et al., 1996). This is sometimes accompanied by methylation of the transgenic DNA (Ingelbrecht et al., 1994; English et al., 1996; Sijen et al., 1996). However, cytosine methylation might not be required for post-transcriptional inactivation, as recently demonstrated in *N. crassa* (Cogoni et al., 1996). Post-transcriptional silencing is often meiotically reversible, shows pronounced developmental and environmental regulation, and may be initiated by an aberrant form of the transgenic RNA and/or by a threshold mechanism (Dehio and Schell, 1994; de Carvalho Niebel et al., 1995; Elmayan and Vaucheret, 1996; English et al., 1996; Goodwin et al., 1996; Kunz et al., 1996; Sijen et al., 1996). There are also a few examples of gene silencing in higher plants in which both transcriptional and post-transcriptional effects have been observed or implied (Dehio and Schell, 1994; Jorgensen, 1995; Jorgensen et al., 1996). The actual molecular processes responsible for epigenetic gene inactivation in plants have not been elucidated yet, in part because several potential silencing mechanisms seem to coexist in most of the cases analyzed.

In organisms with no detectable cytosine methylation of their nuclear genomes, such as Drosophila and yeast (Antequera et al., 1984; Bird, 1992), chromatin structure and nuclear organization seem to play an important role in epigenetic gene silencing (Allshire et al., 1994; Karpen, 1994; Henikoff, 1995; Weiler and Wakimoto, 1995; Elgin, 1996; Felsenfeld, 1996). In Drosophila, euchromatic genes positioned next to heterochromatin by chromosomal rearrangements or P element transposition are frequently silenced in some somatic cells, leading to clonally inherited mosaic patterns of expression. This phenomenon has been termed position effect variegation (PEV) (Karpen, 1994; Henikoff, 1995; Weiler and Wakimoto, 1995; Elgin, 1996). Moreover, some *trans*-acting modifiers of PEV have been shown to encode proteins involved in chromatin formation or modification (Karpen, 1994; Weiler and Wakimoto, 1995; Elgin, 1996). Variegated expression of transgenes integrated into heterochromatic regions has also been observed in mammals (Festenstein et al., 1996; Milot et al., 1996).

Three kinds of hypotheses, namely, somatic elimination of DNA, chromatin packaging, and nuclear compartmentalization, have been proposed to explain the molecular basis of PEV in Drosophila (Karpen, 1994; Henikoff, 1995; Weiler and Wakimoto, 1995; Elgin, 1996). Both the packaging and compartment models postulate that gene inactivation results from the inability of at least part of the transcription machinery to gain access to the DNA. In the packaging model, access is prevented because the chromatin fiber becomes organized into a condensed structure (Karpen, 1994; Wallrath and Elgin, 1995; Elgin, 1996). In contrast, the compartment model postulates that inactivation is due to mislocalization of the variegating gene (perhaps via pairing interactions) into a nuclear subdomain that might lack or prevent interaction with a certain transcription component(s) (Dorer and Henikoff, 1994; Karpen, 1994; Henikoff, 1995; Weiler and Wakimoto, 1995; Sabl and Henikoff, 1996). In many cases, there is not enough information to distinguish among these models, although DNA loss (i.e., somatic elimination) does not seem necessary to mediate PEV in diploid Drosophila tissue (Wallrath et al., 1996).

We have recently constructed a dominant selectable marker for nuclear transformation of Chlamydomonas by placing the coding sequence of the eubacterial *aadA* gene (conferring spectinomycin resistance) under the control of the 5′ and 3′ regulatory regions of the Chlamydomonas *RbcS2* gene (encoding the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase) (Cerutti et al., 1997). This chimeric *RbcS2*::*aadA*::*RbcS2* gene integrates stably into the nuclear genome, predominantly as multiple genetically linked copies (Cerutti et al., 1997). However, its expression is reversibly suppressed in approximately half of the transformants. In this article, we report the detailed analysis of the phenotypic characteristics and the mechanism(s) responsible for this epigenetic phenomenon. Gene silencing was found to result from transcriptional inactivation, but multiple intermediate levels of expression/repression were observed in genetically identical cells. Neither cytosine methylation nor a condensed

chromatin structure that blocks accessibility of the DNA to enzymatic probes appears to be involved in transcriptional repression of the introduced chimeric gene(s). By analogy to the compartment model postulated to explain PEV in Drosophila, we speculate that the different expression states are the consequence of the integrated transgene(s) becoming organized into distinct chromosomal/nuclear domains.

RESULTS

Chlamydomonas Transformants Containing the *RbcS2*::*aadA*::*RbcS2* Gene(s) Integrated into the Nuclear Genome Show an Unstable Spectinomycin-Resistant Phenotype

Using several constructs with varying lengths of the *RbcS2* upstream region driving expression of the eubacterial *aadA* gene, we were able to isolate Chlamydomonas transformants by direct selection on spectinomycin-containing Tris–acetate–phosphate (TAP) plates, as previously described (Cerutti et al., 1997). However, the spectinomycin-resistant (Spc^r) phenotype was unstable in approximately half of the transformants. When subclones of these transformants were cultured for a few weeks under nonselective conditions, their subsequent survival on spectinomycin was significantly reduced. In some cases, after being transferred en masse to spectinomycin-containing TAP plates, only a small percentage of cells survived, giving rise to green patches on a white background of dead cells (Figure 1, transformants 11-P(300), 2-P(650), and 10-P(1030)). In contrast, after being subcultured for several weeks in the absence of spectinomycin, most cells of stable transformants were able to survive when transferred to selective conditions (Figure 1, transformant 10-P(300)).

To examine the loss of spectinomycin resistance quantitatively, subclones of 20 independent transformants, generated with two *RbcS2*::*aadA*::*RbcS2* constructs of different promoter length, P(300) and P(1030) (Cerutti et al., 1997), were grown continuously both in the presence and absence of spectinomycin for 1 month. Subsequently, their Spc^r phenotype was evaluated by single-cell plating on TAP–agar medium containing 75 μg/mL spectinomycin. Forty percent of the transformants displayed an unstable Spc^r phenotype in which the subclones maintained under nonselective conditions yielded less than half the number of spectinomycin-resistant cells found in the subclones kept under selective conditions (Table 1, transformants with inactivation factor $\geq$2). However, the degree of phenotypic instability varied considerably, with two transformants showing a >1000-fold reduction in survival on spectinomycin and others only displaying decreases ranging from two- to 5.4-fold (Table 1).

There was no obvious relationship between the instability of the Spc^r phenotype and the number of copies of integrated transforming DNA or the construct used for transformation (Table 1). However, the stability of the Spc^r phenotype did correlate with the time that transformants appeared as visible colonies on the selective plates after transformation. As previously described (Cerutti et al., 1997), transformants were obtained by microprojectile bombardment or glass bead agitation of cells followed by selection for 3 weeks on TAP–agar plates containing 90 μg/mL spectinomycin. Most transformants identified as visible colonies during the first 7 to 10 days of incubation showed a stable Spc^r phenotype (Table 1, fast-growing transformants). In contrast, the majority of transformants that required 10 to 21 days to form a visible colony on the selective plates displayed an unstable Spc^r phenotype (Table 1, slow-growing transformants).

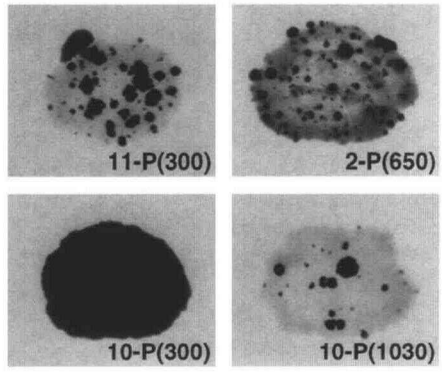


Figure 1. Stable and Unstable Spc^r Phenotypes of Chlamydomonas Transformants Containing the *RbcS2*::*aadA*::*RbcS2* Gene(s) Integrated into the Nuclear Genome.

After being selected on TAP plates containing spectinomycin, transformants were grown for 4 weeks under nonselective conditions. Cells were then transferred en masse to selective plates, spread to form a patch, and incubated for 2 weeks at 25°C. Dark areas correspond to surviving cells that divide to produce green sectors, whereas gray areas indicate dead cells. One transformant with a stable Spc^r phenotype, 10-P(300), and three transformants with unstable phenotypes are shown. The numbers between parentheses in the transformant designations indicate the length in base pairs of the *RbcS2* upstream sequence used as promoter in the constructs (Cerutti et al., 1997).

The Spc^r and Spectinomycin-Sensitive Phenotypes of Chlamydomonas Transformants Are Reversible without Detectable Alterations of the Integrated *RbcS2*::*aadA*::*RbcS2* Gene(s)

Several mechanisms can be envisaged to explain the phenotypic instability of Chlamydomonas nuclear transformants. These mechanisms can be broadly classified into two categories: (1) those resulting in alterations of the nucleotide sequence of the introduced *RbcS2*::*aadA*::*RbcS2*

Table 1. Spectinomycin Resistance of Chlamydomonas Transformants Containing the *RbcS2::aadA::RbcS2* Gene(s) Integrated into the Nuclear Genome

Transformant	Copy Number[a]	Cell Survival on Spectinomycin (%)[b]		Inactivation Factor (+/−)[d]
		−[c]	+[c]	
Fast growing[e]				
6-P (300)	2	91.9	99.7	1.1
7-P (300)	1.5	91.8	99.9	1.1
8-P (300)	3	44.6	93.8	2.1
9-P (300)	2	90.5	97.7	1.1
10-P (300)	4	97.5	96.5	1.0
15-P (1030)	2.5	85.8	99.8	1.2
16-P (1030)	2	79.6	99.2	1.2
17-P (1030)	3.5	93.6	100.0	1.1
18-P (1030)	13	99.4	98.1	1.0
19-P (1030)	1	97.6	99.6	1.0
Slow growing[f]				
21-P (300)	2	35.8	94.9	2.7
22-P (300)	9	0.02	55.1	2755.0
23-P (300)	2	71.4	100.0	1.4
24-P (300)	1.5	58.3	96.7	1.7
25-P (300)	5	17.3	93.1	5.4
26-P (1030)	1.5	40.7	91.4	2.2
27-P (1030)	1	0.02	68.0	3400.0
28-P (1030)	6	94.1	97.8	1.0
29-P (1030)	3	21.6	90.0	4.2
30-P (1030)	2	37.5	76.1	2.0

[a] Estimated number of copies of the *RbcS2::aadA::RbcS2* gene(s) integrated into the nuclear genome.

[b] [(Number of colonies on selective plates)/(number of colonies on nonselective plates)] × 100 (see Methods for details).

[c] Subclones grown for 4 weeks as streaks on TAP plates (−) or spectinomycin-containing TAP plates (+) before determination of cell survival on spectinomycin.

[d] Ratio of cell survival on spectinomycin of subclones maintained under selective conditions versus subclones kept in the absence of selection.

[e] Transformants forming a visible colony within 10 days of transformation.

[f] Transformants forming a visible colony 10 to 21 days after transformation.

gene(s) (i.e., genetic changes), such as deletions, rearrangements, and/or mutations; and (2) those leading to gene inactivation without modifications of the DNA sequence (i.e., epigenetic changes). If genetic modifications were responsible for the loss of antibiotic resistance, spectinomycin-sensitive subclones would be expected to be phenotypically very stable and perhaps show alterations in the integrated foreign DNA detectable by gel blot hybridization. Moreover, in the case of multiple-copy transformants, changes in the nucleotide sequence would have to occur concomitantly in all functional copies of the introduced *RbcS2::aadA::RbcS2* gene, because the resistance phenotype is dominant. In contrast, if the spectinomycin-sensitive (Spc^s) phenotype resulted from epigenetic changes, then spectinomycin-sensitive cells might be expected to revert at rates significantly higher than the nuclear mutation frequency and to show no accompanying changes in the integrated foreign gene(s). To distinguish between these possibilities, we performed a clonal pedigree analysis of representative transformants (Figure 2A).

Chlamydomonas transformants most likely arise by integration of the *RbcS2::aadA::RbcS2* gene(s) into the nuclear genome of a single cell that becomes spectinomycin resistant and divides, forming a colony on selective plates (Bishop and Smith, 1989; Cerutti et al., 1997). Therefore, all cells in a subsequent culture derived from this colony are in principle genetically identical. After their original selection (Figure 2A, phase I), several independent transformants were propagated as streaks on nonselective TAP plates for 4 weeks (Figure 2A, phase II). Three transformants (11-P(300), 1-P(1030), and 2-P(650)) were chosen for detailed characterization because they have different lengths of the *RbcS2* promoter sequence (as indicated by the numbers between parentheses given in base pairs) driving expression of the *aadA* gene, and they contain widely different numbers of copies of integrated transforming DNA (Figure 2C). After 4 weeks under nonselective conditions, approximately half of the streak culture from each transformant was transferred to and maintained on spectinomycin-containing TAP plates for another 8 weeks, whereas the other half was propagated similarly in the absence of selection (Figure 2A, phase III). At the end of this 8-week period, the subclones maintained under selective conditions showed significantly greater survival on spectinomycin than did the ones grown continuously in the absence of the antibiotic (Figure 2B; cf. subclones designated [−] and [+]). However, DNA gel blot analysis demonstrated that there were no detectable differences in the hybridization patterns of the integrated transforming DNA between these subclones (Figure 2C, subclones designated [−] and [+]), except for a minor rearrangement in the multiple-copy transformant 2-P(650) (Figure 2C, arrowheads) that apparently does not correlate with the Spc^r phenotype (see below).

To examine whether the Spc^r and the Spc^s phenotypes were reversible, cells from the different subclones were plated at low density, to obtain individual colonies, on the same kind of medium on which they had been propagated (Figure 2A, phase IV). Subsequently, cells from 50 individual colonies were independently transferred to the opposite selective condition (i.e., colonies from subclones grown in the absence of spectinomycin were transferred to TAP–agar plates containing spectinomycin, and vice versa) (Figure 2A, phase V). Because the cell survival on spectinomycin of subclones 11-P(300) [−] and 2-P(650) [−] is <2% (Figure 2B), only one of the 50 colonies might be expected to be formed by a spectinomycin-resistant cell. Thus, when transferring

from nonselective to selective conditions (Figure 2A, phase V, lower half), only one colony from those subclones would be expected to grow on the antibiotic-containing plates. In the same way, ~50% of the colonies from subclone 1-P(1030) [−] should survive the transfer to selective plates. In addition, most or all cells from these colonies should be spectinomycin resistant and therefore form a uniformly green patch on selective medium (similarly to transformant 10-P(300) in Figure 1).

In contrast to these expectations, a much higher percentage of the 50 colonies from the subclones maintained under nonselective conditions were able to survive the transfer to selective plates (Figure 2D). Moreover, in most cases, only a small fraction of the transferred cells from each colony survived on spectinomycin-containing plates, giving rise to green patches on a background of dead cells (similar to the unstable spectinomycin-resistant transformants in Figure 1). These observations indicated that the founder cells of many colonies were spectinomycin sensitive, but during the mitotic divisions required to form a visible colony on the nonselective plates (Figure 2A, phase IV, lower half), some cells were able to revert back to an Spc^r phenotype. Four surviving colonies from each transformant, chosen at random, were maintained under selective conditions for an additional 12 weeks (Figure 2A, phase VI, lower half; subclones e to h) and then evaluated for cell survival on spectinomycin (see below).

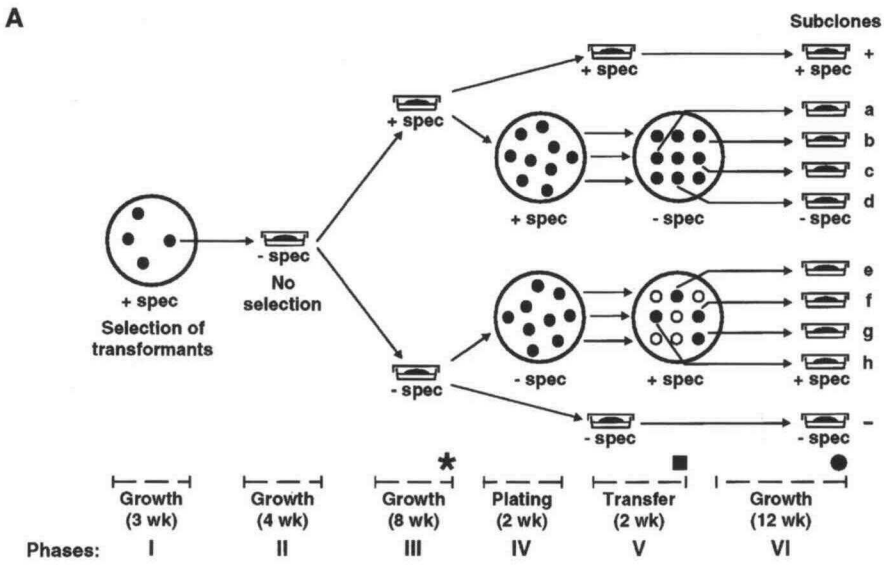
When 50 colonies from subclones cultured under selective conditions were transferred to nonselective conditions (Figure 2A, phase V, upper half), all colonies survived, as expected. To test whether cells from these spectinomycin-resistant colonies can revert to spectinomycin sensitivity, four colonies from each transformant were randomly selected and propagated as individual subclones in the absence of spectinomycin for 12 more weeks (Figure 2A, phase VI, upper half; subclones a to d).

All subclones, including the original ones kept under constant selective or nonselective conditions (Figure 2A, phase VI, subclones designated [−] and [+]), were evaluated for cell survival on spectinomycin after the 12-week period (Table 2). Subclones continuously maintained in the absence of antibiotic showed a decrease in survival when tested on spectinomycin (cf. Figure 2B and Table 2, subclones designated [−]). Conversely, subclones kept continuously under selective conditions yielded a greater percentage of spectinomycin-resistant cells than they did 4 months earlier (cf. Figure 2B and Table 2, subclones designated [+]). However, these subclones did not achieve complete resistance, suggesting that some spectinomycin-resistant cells can revert to being spectinomycin sensitive. Most subclones of individual colonies transferred from nonselective to selective conditions and maintained under selection for 3 months behaved in similar fashion (Table 2, subclones e to h). Moreover, subclones of individual colonies transferred from selective to nonselective conditions and propagated in the absence of spectinomycin for 3 months showed significantly <100% survival on spectinomycin (Table 2, subclones a to d). Because these subclones derive from single cells that were spectinomycin resistant (the founders of the spectinomycin-resistant colonies in Figure 2A, phase IV, upper half), these observations clearly indicate that some spectinomycin-resistant cells can revert to a Spc^s phenotype.

DNA gel blot analysis demonstrated that the Spc^r and Spc^s phenotypes of a given transformant (Table 2) did not correlate with any detectable alterations of the integrated transforming DNA (Figure 2E). Some minor rearrangements were observed in subclones of transformant 2-P(650) (Figure 2E, subclones b, c, and h). However, these subclones were about as resistant to spectinomycin as subclone g (Table 2), which showed no detectable rearrangement (Figure 2E), consistent with the lack of relationship between the Spc^r phenotype and minor alterations of the integrated foreign DNA. Moreover, several 2-P(650) subclones (e.g., subclones e to g and [−]) appear identical on DNA gel blots (Figure 2E) but differ substantially in their cell survival on spectinomycin (Table 2).

We also examined the inheritance of the integrated foreign DNA and the Spc^r phenotype in the progeny of sexual crosses between selected transformants and the spectinomycin-sensitive wild-type strain CC-125. For these crosses, we used the subclones that had been propagated for 4 weeks in the absence of spectinomycin after transformation and then maintained continuously under selective conditions (Figure 2A, subclones designated [+]). All products of 10 complete tetrads for each transformant (11-P(300), 1-P(1030), and 2-P(650)) crossed to the wild-type strain CC-125 were analyzed by DNA gel blotting (Figure 3A; data not shown). As previously demonstrated (Cerutti et al., 1997), the transforming DNA displayed Mendelian inheritance, confirming its stable integration into the nuclear genome. The single-copy transformant 11-P(300) showed a straightforward 2:2 segregation for both the integrated *aadA* gene and the Spc^r phenotype (Figure 3A). Transformant 1-P(1030) contained ~5.5 copies of the *RbcS2::aadA::RbcS2* gene that cosegregated as a single genetic locus (Figure 3A), whereas transformant 2-P(650) contained ~100 copies integrated at two loci that segregated independently (data not shown; Cerutti et al., 1997). In all cases, the Spc^r phenotype cosegregated with the *RbcS2::aadA::RbcS2* gene(s) (Figures 3A and 3B; Cerutti et al., 1997), indicating that in cells reselected after 4 weeks of propagation in the absence of spectinomycin, the antibiotic resistance phenotype is conferred by the integrated chimeric gene(s). In contrast, spontaneous spectinomycin resistance in Chlamydomonas results mainly from mutations in the chloroplast 16S rRNA gene (Harris, 1989), and it occurs at a very low frequency ($<10^{-7}$) (Wurtz et al., 1979; Cerutti et al., 1997).

In summary, the clonal pedigree analysis demonstrated that (1) both the Spc^r and Spc^s phenotypes are reversible; (2) these changes occur at a frequency considerably higher than that expected for conventional genetic mutation; and



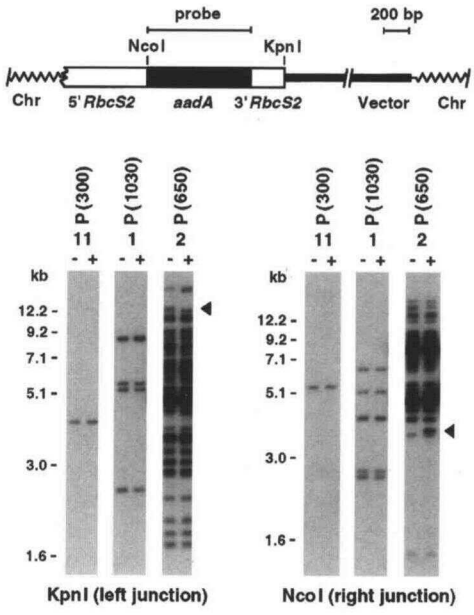
B ✱ End of Phase III

Transformant	Cell survival on spectinomycin (%)	
	-	+
11-P(300)	0.2	68.3
1-P(1030)	51.2	92.7
2-P(650)	1.8	65.7

D ■ End of Phase V

Subclone [-] of transformant	Colony survival on spectinomycin (%)
11-P(300)	40.0
1-P(1030)	100.0
2-P(650)	34.0

C ✱ End of Phase III



E ● End of Phase VI

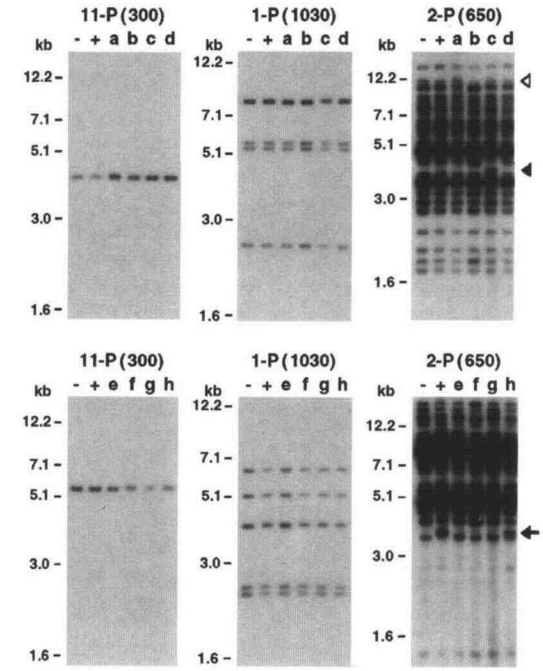


Figure 2. Clonal Pedigree Analysis of Chlamydomonas Transformants with an Unstable Spc^r Phenotype.

(3) the phenotypic interconversions do not involve any obvious alteration of the integrated transforming DNA. Moreover, the genetic crosses indicated that the Spc^r phenotype cosegregated with the integrated *RbcS2*::*aadA*::*RbcS2* gene(s), ruling out the possibility that antibiotic resistance might result from the selection of spontaneous mutants unrelated to the introduced selectable marker. Taken together, these results are consistent with an epigenetic mechanism(s) being responsible for the silencing of the integrated *RbcS2*::*aadA*::*RbcS2* gene(s).

The Introduced *RbcS2*::*aadA*::*RbcS2* Gene(s) Is Transcriptionally Repressed

To understand the molecular basis of the observed Spc^r and Spc^s phenotypes, we examined expression of the *RbcS2*::*aadA*::*RbcS2* gene(s) by evaluating mRNA and protein levels in representative transformants. On RNA gel blots, the *RbcS2*::*aadA*::*RbcS2* transcript was clearly observed in subclones kept under selection (Table 3; Cerutti et al., 1997). However, it was undetectable or present at a reduced level in subclones maintained under nonselective conditions. The steady state level of the mRNA correlated with the steady state level of the immunologically detected AadA protein (Cerutti et al., 1997). Because the integrated transforming DNA was not altered or lost (see above), these results supported the interpretation that a silencing mechanism(s) was preventing expression of the *RbcS2*::*aadA*::*RbcS2* gene(s) in the subclones propagated under nonselective conditions.

The reduced accumulation of *RbcS2*::*aadA*::*RbcS2* mRNA could result from decreased transcription and/or decreased transcript stability in subclones maintained in the absence of selection. To distinguish between these possibilities, we estimated the transcription rate of the introduced *RbcS2*::*aadA*::*RbcS2* gene(s) by nuclear run-on assays. Nuclei were isolated from subclones of transformants 11-P(300), 1-P(1030), and 2-P(650) kept under selective or nonselective conditions. Nuclear run-on transcripts corresponding to the *aadA* coding sequence were clearly detected in nuclei from subclones propagated in the presence of spectinomycin (Figure 4A). However, they were present at a reduced level (up to 35-fold lower for transformant 2-P(650)) in nuclei from subclones maintained under nonselective conditions (Figure 4B and Table 3). In contrast, similar levels of nuclear run-on transcripts for two endogenous Chlamydomonas genes, *TubA* (encoding α_1-tubulin) and *RbcS*, were found in nuclei from both types of subclones (Figure 4A).

The transcriptional activity of the introduced *RbcS2*::*aadA*::*RbcS2* gene(s) correlated with the steady state level of the transcripts normalized in both cases to the endogenous *RbcS2* gene (Table 3; data not shown). Moreover, as already mentioned, there was a direct relationship between the mRNA and the protein levels corresponding to the *RbcS2*::*aadA*::*RbcS2* gene(s) (Cerutti et al., 1997). Therefore,

Figure 2. (continued).

(A) Strategy used for clonal pedigree analysis of unstable spectinomycin-resistant transformants (see text for details). For propagation, cells were transferred every 2 weeks to fresh TAP plates (− spec) or spectinomycin-containing TAP plates (+ spec). Subclones of the transformants were analyzed for cell survival on spectinomycin and for rearrangements of the integrated foreign DNA at the times indicated by an asterisk (results shown in **[B]** and **[C]**) and by a closed circle (results shown in **[E]** and Table 2). Colony survival on spectinomycin was evaluated at the time indicated by a closed square (results shown in **[D]** for subclones designated [−]).

(B) Cell survival on spectinomycin of subclones of the indicated transformants maintained under nonselective (−) or selective (+) conditions for 8 weeks (asterisk in **[A]**). Survival was determined by counting colonies 10 days after spreading the cells on TAP plates with and without spectinomycin, as described in Methods.

(C) Diagram of one copy of the integrated *RbcS2*::*aadA*::*RbcS2* plasmid(s) and DNA gel blot analysis of subclones of the indicated transformants grown in the absence (−) or presence (+) of spectinomycin for 8 weeks (asterisk in **[A]**). Total cell DNA was digested with KpnI or NcoI and probed with the coding sequence of *aadA*, detecting the left or right junctions, respectively, of plasmid DNA with Chlamydomonas chromosomal (Chr) DNA (as described in Cerutti et al., 1997). The arrowheads indicate minor rearrangements of the integrated foreign DNA in transformant 2-P(650). Numbers at left indicate the positions of molecular weight DNA markers in kilobases.

(D) Cells from each of 50 individual colonies (phase IV in **[A]**) of subclones of the indicated transformants, maintained under nonselective conditions, were transferred to spectinomycin-containing TAP plates and spread, forming a small patch. After 2 weeks of incubation (closed square in **[A]**), a colony was scored as surviving if some cells on the patch were able to grow and form green sectors.

(E) DNA gel blot analysis of the indicated subclones (closed circle in **[A]**) to examine the existence of deletions/rearrangements of the integrated *RbcS2*::*aadA*::*RbcS2* gene(s). Numbers at left indicate the positions of molecular weight DNA markers in kilobases. The three upper gel blots (KpnI digestions) show the left junction of the integrated plasmid(s) with chromosomal DNA, as described in **(C)**. The open and closed arrowheads indicate very small rearrangements of the introduced chimeric genes in subclones b and c, respectively, of the multiple-copy transformant 2-P(650). The three lower gel blots (NcoI digestions) show the right junction of the integrated plasmid(s) with chromosomal DNA, as described in **(C)**. The arrow indicates a minor rearrangement of the introduced *RbcS2*::*aadA*::*RbcS2* plasmids in subclone h of transformant 2-P(650).

Table 2. Spectinomycin Resistance of Subclones of Unstable Spectinomycin-Resistant Chlamydomonas Transformants Subjected to Clonal Pedigree Analysis[a]

	Cell Survival on Spectinomycin (%)[b]		
Subclones	11-P (300)	1-P (1030)	2-P (650)
+[c]	92.8	94.9	84.6
a[d]	43.8	77.4	27.1
b[d]	55.3	89.1	70.3
c[d]	76.9	82.3	63.8
d[d]	60.2	75.8	45.2
e[e]	50.4	83.7	16.3
f[e]	79.3	86.3	8.1
g[e]	92.5	100.0	60.3
h[e]	88.1	76.9	63.5
−[f]	0.05	44.7	0.5

[a] See Figure 2A, end of phase IV.
[b] See Table 1, footnote b.
[c] Subclones maintained under selective conditions (Figure 2A).
[d] Subclones from individual colonies transferred from selective to nonselective conditions (Figure 2A).
[e] Subclones from individual colonies transferred from nonselective to selective conditions (Figure 2A).
[f] Subclones maintained under nonselective conditions (Figure 2A).

taken together, our results demonstrate that expression of the introduced *RbcS2*::*aadA*::*RbcS2* gene(s) is transcriptionally suppressed by an epigenetic mechanism(s).

Genetically Identical Cells Appear to Express the Introduced *RbcS2*::*aadA*::*RbcS2* Gene(s) at Multiple Different Levels That Are Mitotically Metastable

Although Chlamydomonas transformants containing the *RbcS2*::*aadA*::*RbcS2* gene(s) displayed reversible resistance to spectinomycin, the phenotypes of the colonies were not consistent with a simple on/off switch between two alternate, spectinomycin-resistant and spectinomycin-sensitive states. Under nonselective conditions, colonies of the 11-P(300), 1-P(1030), and 2-P(650) transformants were fairly uniform in size (Figure 5A). However, in the presence of spectinomycin, all transformants showed heterogeneity in colony size (Figures 5A and 5C; data not shown), suggesting that genetically identical cells forming independent colonies have variable levels of expression of the introduced chimeric gene(s) and therefore different degrees of antibiotic resistance. In contrast, nuclear transformants with a stable Spc^r phenotype formed colonies of homogeneous size on selective plates (data not shown). In addition, subclones derived from individual cells of a given transformant showed different levels of cell survival on spectinomycin, although they were kept under continuous selection for 12 weeks (Table 2, subclones e to h). These observations support the existence of multiple, apparently metastable, expression states in individual cells (see below).

Because transcription of the integrated *RbcS2*::*aadA*::*RbcS2* gene(s) correlates with the steady state levels of the corresponding mRNA and protein (Table 3; Cerutti et al., 1997), the resistance to spectinomycin of individual cells most

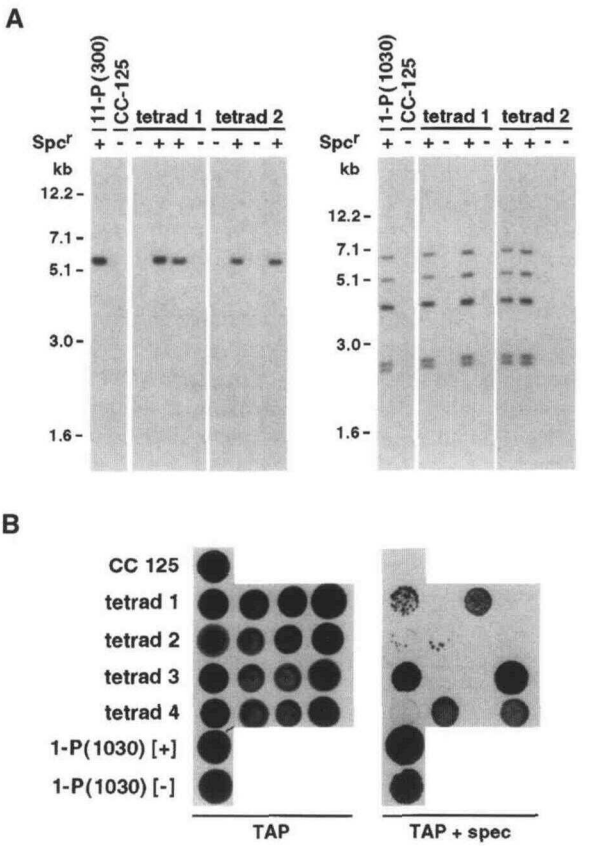


Figure 3. Cosegregation of the Spc^r Phenotype and the *RbcS2*::*aadA*::*RbcS2* Gene(s) among the Progeny of Sexual Crosses of Chlamydomonas Transformants 11-P(300) and 1-P(1030) with the Spectinomycin-Sensitive Wild-Type Strain CC-125.

(A) DNA gel blots (NcoI digestion as described in Figure 2C) of the parental strains and the products of two complete tetrads from the crosses of the two transformants. Numbers at left indicate the positions of molecular weight DNA markers in kilobases.

(B) Growth and survival in the absence (TAP) or presence (TAP + spec) of spectinomycin of the parental strains and the products of four complete tetrads from the cross of transformant 1-P(1030) (Figure 2A, subclone designated [+]). The 1-P(1030) [−] subclone was also included to compare its Spc^r phenotype with that of the tetrad products. Cells grown mixotrophically to middle log phase were diluted in TAP medium to 5×10^3 cells per 5 μL and spotted on the plates. Shown is the growth of the cells after 10 days of incubation, as described in Methods.

Table 3. Transcriptional Activity and mRNA Level of the Integrated *RbcS2*::*aadA*::*RbcS2* Gene(s) and Cell Survival on Spectinomycin of Unstable Spectinomycin-Resistant Chlamydomonas Transformants Maintained under Nonselective or Selective Conditions

Transformant[a]	Copy Number[b]	Unmethylated Copies[c]	Relative Transcription (%)[d]	Relative mRNA Level (%)[e]	Cell Survival on Spectinomycin (%)[f]
11-P (300) [−]	1	1	5	ND[g]	0.05
11-P (300) [+]	1	1	100	100	92.8
1-P (1030) [−]	5.5	1.5	100	80	44.7
1-P (1030) [+]	5.5	1.5	220	200	94.9
2-P (650) [−]	100	13	20	ND	0.5
2-P (650) [+]	100	13	750	790	84.6

[a] Subclones of the indicated transformants were maintained under nonselective (−) or selective (+) conditions (Figure 2A, phase VI).
[b] Estimated number of copies of the *RbcS2*::*aadA*::*RbcS2* gene(s) integrated into the nuclear genome.
[c] Number of unmethylated copies estimated as described in Methods.
[d] Relative transcription determined as explained in the legend to Figure 6.
[e] The *aadA* mRNA level of the different subclones was normalized to that of the endogenous *RbcS2* gene. The mean of the 11-P (300) [+] subclone was arbitrarily set to 100%, and all of the other values were adjusted accordingly. The steady state level of the *RbcS2*::*aadA*::*RbcS2* mRNA was very low, even in subclones maintained under selective conditions (Cerutti et al., 1997).
[f] See Table 1, footnote b.
[g] ND, not detectable. Below the limit of detection in gel or slot blots of total RNA.

likely reflects their transcriptional activity. Therefore, cell survival on spectinomycin indirectly mirrors gene transcription in single cells. If only two alternate expression states were possible (Figure 5B, On/Off States), all cells surviving on spectinomycin-containing plates would transcribe the chimeric gene(s) to its fullest extent. Conversely, cells unable to survive under selective conditions would be transcriptionally silent. According to this on/off model, the relative level of transcription for a given transformant should be approximately equal to its relative survival on spectinomycin. Alternatively, individual cells could express the *RbcS2*::*aadA*::*RbcS2* gene(s) to different degrees (Figure 5B, Intermediate States), but only those transcribing above a certain threshold would be able to survive on spectinomycin-containing plates. In this model, the relative level of transcription should be higher than the relative survival on spectinomycin because some cells transcribing the chimeric gene(s) will be unable to survive the selection.

The validity of the on/off model can be examined using the data summarized in Table 3. According to this hypothesis, normalizing the transcription and cell survival on spectinomycin of subclones kept under nonselective conditions to those of subclones maintained under continuous selection (theoretically transcribing the chimeric gene[s] to maximal level in nearly all cells), the relative values obtained should be approximately equal (i.e., transcriptional activity in a subclone designated [−] divided by transcriptional activity in a subclone designated [+] should equal approximately cell survival on spectinomycin of a subclone designated [−] divided by cell survival on spectinomycin of a subclone designated [+]). An examination of Table 3 reveals that for transformants 11-P(300) and 2-P(650), the relative transcription is significantly higher than the relative survival on spectinomycin. Therefore, our results are not compatible with the existence of only two alternate (on/off) transcriptional states but are consistent with multiple different expression states. In the case of transformant 1-P(1030), the reduction in transcriptional activity in subclones kept under nonselective conditions is too small to discriminate between the models. The analysis of the data in relative terms corrects for the fact that the maximal level of transcriptional activity in some transformants might be higher than that required to survive on 75 μg/mL spectinomycin (Table 3; cf. 2-P(650) [+] and 11-P(300) [+]).

The existence of intermediate expression states of the introduced *RbcS2*::*aadA*::*RbcS2* gene(s) is also supported by the results of survival tests at different concentrations of spectinomycin. Plating experiments of subclone 11-P(300) [−] yielded increasing numbers of surviving cells as the spectinomycin concentration was reduced from 90 to 60 μg/mL (Figure 5C). Because even the lowest spectinomycin concentration is still lethal for wild-type Chlamydomonas, these observations are consistent with genetically identical cells of a given transformant being able to express the selectable marker at different levels. Moreover, at the lowest selective pressure, there was marked heterogeneity in colony size (Figure 5C). In contrast, as the selective pressure increased, the small colonies progressively disappeared, most likely because their founder cells did not express the introduced chimeric gene(s) at a level high enough to be able to survive and form a visible colony (Figure 5C).

A certain expression state, once established, appears to be mitotically metastable for multiple cell generations. For instance, if a cell in a colony under selection were to switch to a higher level of expression, the progeny from that cell would be able to overgrow the neighboring cells, resulting in a

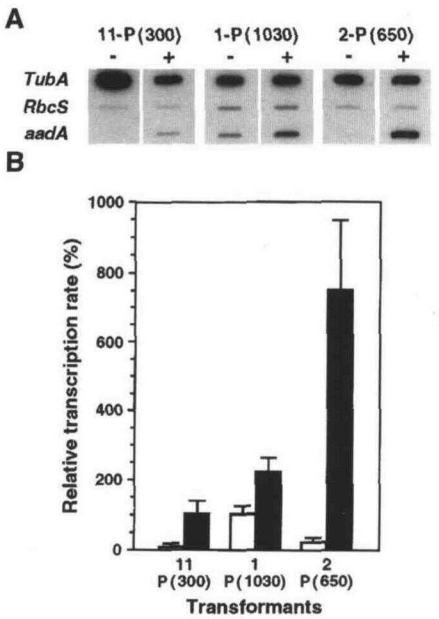


Figure 4. The Introduced *RbcS2*::*aadA*::*RbcS2* Gene(s) Is Transcriptionally Silenced.

(A) Transcriptional activity of the chimeric *RbcS2*::*aadA*::*RbcS2* gene(s) in nuclear run-on assays of the indicated transformants maintained under nonselective (−) or selective (+) conditions. Transcription of the endogenous Chlamydomonas genes *TubA* and *RbcS* was evaluated as a control (see text for details).

(B) Relative transcription rate of the *RbcS2*::*aadA*::*RbcS2* gene(s) in the different transformants. The transcriptional activity of subclones maintained in the absence of selection (open bars) or under selection (closed bars) was normalized to that of the endogenous *RbcS* gene. Values shown are the mean ±SE of three independent experiments. The mean of the 11-P(300) [+] subclone was arbitrarily set to 100%, and all of the other values were adjusted accordingly.

lobed colony. Conversely, if a cell were to switch to a lower level of expression, its progeny would grow more slowly than surrounding cells, resulting in a scallop-shaped colony. This switching between expression states does not appear to occur very often, because we observed predominantly well-rounded colonies, albeit quite variable in size (Figures 5A and 5C; data not shown). Because these colonies contain at least 10^5 to 10^6 cells (Randolph-Anderson et al., 1993), the different expression states appear to be stable for at least 15 to 20 generations at 25°C. Consistent with this interpretation, the frequency of reversion to spectinomycin resistance of the 11-P(300) [−] subclone was estimated to be $\sim 5 \times 10^{-5}$ per cell per generation in preliminary experiments.

Although the various expression states seem to be mitotically metastable, they are less stable meiotically (Figure 3B; data not shown). After crossing transformants 11-P(300), 1-P(1030), and 2-P(650) with the spectinomycin-sensitive wild-type strain CC-125 (see above), many tetrad products carrying the introduced *RbcS2*::*aadA*::*RbcS2* gene(s) showed greatly reduced survival on spectinomycin (Figure 3B; data not shown). Four representative tetrads from the cross of transformant 1-P(1030) are shown in Figure 3B (cf. the tetrad products with the parental subclone 1-P(1030) [+]). These results are unlikely to be the result of segregation of a modifier of transgene expression/repression present in the wild-type strain CC-124 used for transformation, because in crosses of stable spectinomycin-resistant transformants, all meiotic products containing the *RbcS2*::*aadA*::*RbcS2* gene(s) displayed levels of spectinomycin resistance similar to that of the parental subclone (data not shown).

In summary, our observations suggest that genetically identical cells of a given transformant appear to express the introduced *RbcS2*::*aadA*::*RbcS2* gene(s) at multiple different levels that are mitotically metastable but less stable meiotically. In fact, the inactive state(s) (operationally defined as an expression level below the threshold required for survival on TAP plates containing 75 μg/mL spectinomycin) seems more stable than do the active states, not only meiotically but also mitotically. Cells cultured under nonselective conditions progressively tend toward a predominantly Spc^s phenotype, although most transformants are not completely silenced and some cells are capable of reverting to spectinomycin resistance.

Transcriptional Inactivation of the *RbcS2*::*aadA*::*RbcS2* Gene(s) Does Not Correlate with Cytosine Methylation of the Introduced DNA

An inverse correlation between gene activity and cytosine methylation, particularly of the promoter and coding regions, has been reported in several eukaryotes (Eden and Cedar, 1994; Rossignol and Faugeron, 1994; Martienssen and Richards, 1995; Singer and Selker, 1995; Hohn et al., 1996; Meyer and Saedler, 1996). This prompted us to examine whether DNA methylation was responsible for the transcriptional silencing of the *RbcS2*::*aadA*::*RbcS2* gene(s) in Chlamydomonas transformants. The methylation of specific cytosine residues in the *aadA* coding sequence and the *RbcS2* promoter region (Figure 6A) was determined using a pair of isoschizomeric restriction enzymes, HpaII and MspI, that recognize the same target sequence (CCGG) but differ in their sensitivity to cytosine methylation. HpaII does not cleave if either one or both cytosines in the recognition site are methylated, whereas MspI is able to cleave if only the internal C residue is methylated (Nelson and McClelland, 1991).

Ten representative transformants, including the ones previously used for pedigree analysis and nuclear run-on assays, were examined by gel blot hybridization of total cell DNA digested with HindIII and MspI or HindIII and HpaII and probed with the coding sequence of *aadA*. In our samples, HindIII cleaved to completion independently of methylation status (data not shown). In the absence of methylation, the probe would detect a fragment of 530 bp (arrowheads in

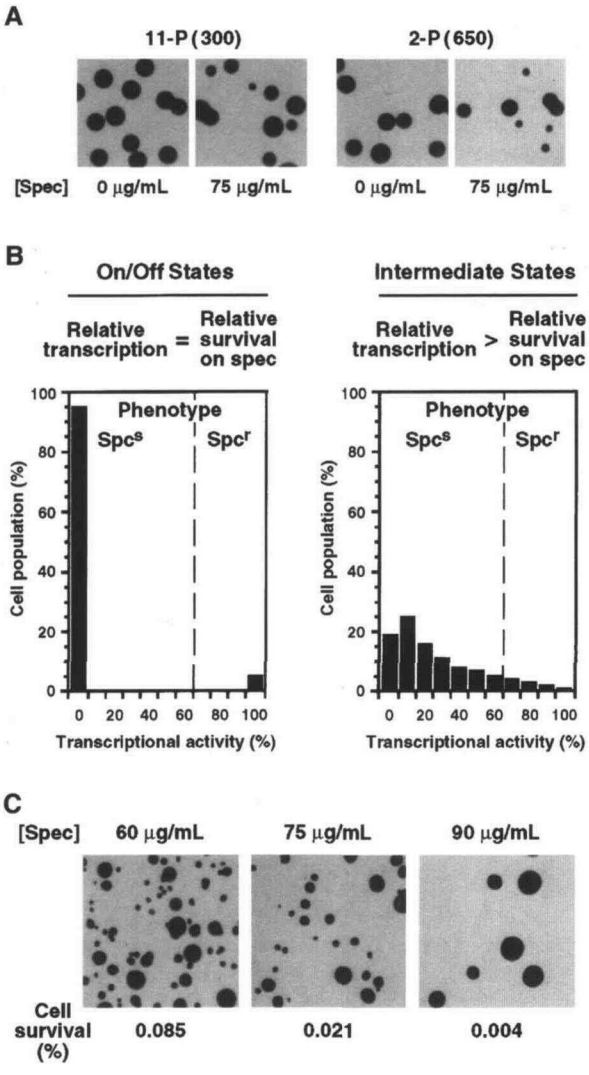


Figure 5. Individual Transformed Cells Seem to Express the Introduced *RbcS2*::*aadA*::*RbcS2* Gene(s) at Different Intermediate Levels That Are Mitotically Metastable.

(A) Subclones of transformants 11-P(300) and 2-P(650) maintained continuously under selective conditions (Figure 2A, subclones designated [+]) form colonies of fairly uniform size when plated under nonselective conditions but of very heterogeneous size when spread on plates containing 75 μg/mL spectinomycin (Spec). This suggests that genetically identical cells forming different colonies express the introduced *RbcS2*::*aadA*::*RbcS2* gene at different levels.

(B) Models of expression of the introduced *RbcS2*::*aadA*::*RbcS2* gene(s) in individual cells of unstable spectinomycin-resistant Chlamydomonas transformants. The existence of intermediate expression states is consistent with several lines of evidence (see text for details). The dashed lines indicate the minimal level of expression required for cell survival on a given concentration of spectinomycin (spec).

(C) In support of the intermediate states model shown in **(B)**, the percentage of cells surviving on spectinomycin (Spec) increases as the concentration of antibiotic in the plates decreases (see text for details). Approximately 5×10^5 cells of the 11-P(300) [−] subclone (Figure 2A) were spread on TAP–agar plates containing the indicated concentrations of spectinomycin. Cell survival on spectinomycin was estimated by counting visible colonies after 15 days of incubation.

Figure 6B) and three fragments <160 bp (Figure 6A). These three small segments were run off the gels shown in Figures 6B and 6C. As some of the sites become methylated, failure of HpaII or MspI to digest would result in fragments of higher molecular weight. Our analysis of five independent single-copy transformants revealed no detectable methylation in the *RbcS2* upstream sequence or in the *aadA* coding sequence (Figure 6B, transformant 11-P(300); data not shown). However, there was extensive methylation of the introduced *RbcS2*::*aadA*::*RbcS2* genes in multiple-copy transformants (Figure 6B, transformants 1-P(1030) and 2-P(650)). Because MspI was able to digest much more extensively than HpaII, these results also indicated that in Chlamydomonas, methylation of cytosine residues within CG dinucleotides is more frequent than methylation in CNG trinucleotides (Figure 6B, transformants 1-P(1030) and 2-P(650); cf. HindIII and MspI gels, and HindIII and HpaII gels).

Using additional methylation-sensitive restriction endonucleases such as HhaI, ScrFI, and SmaI, we examined 40% of the cytosine residues in the 180-bp region immediately upstream of the transcription start. In no case did we detect methylation of the introduced *RbcS2*::*aadA*::*RbcS2* gene in single-copy transformants, whereas methylation was marked in multiple-copy transformants (data not shown). Complete digestion of total cell DNA by methylation-sensitive restriction enzymes was checked by rehybridization of the filters with the coding sequence of the chloroplast *chlL* gene (encoding a subunit of the light-independent protochlorophyllide oxidoreductase, required for the synthesis of chlorophyll in the dark) (data not shown), which is present at an ~80-fold higher copy number than are single-copy nuclear genes (Harris, 1989).

Although our results clearly demonstrate that the introduced DNA is extensively methylated in multiple-copy transformants, this DNA modification did not correlate with transcriptional inactivation of the *RbcS2*::*aadA*::*RbcS2* genes. Transformants 1-P(1030) and 2-P(650) showed similar degrees of cytosine methylation in the subclones maintained under nonselective conditions as in those kept continuously under selection (Figure 6B; cf. subclones designated [−] and [+]). However, transcription of the *RbcS2*::*aadA*::*RbcS2* genes differed substantially in these subclones (Figure 4 and Table 3). Moreover, the single-copy transformant 11-P(300) showed an ~20-fold reduction in transcription when maintained under nonselective conditions (Figure 4 and Table 3) without any detectable methylation of the integrated *RbcS2*::*aadA*::*RbcS2* gene (Figure 6B; data not shown). We also analyzed methylation in the

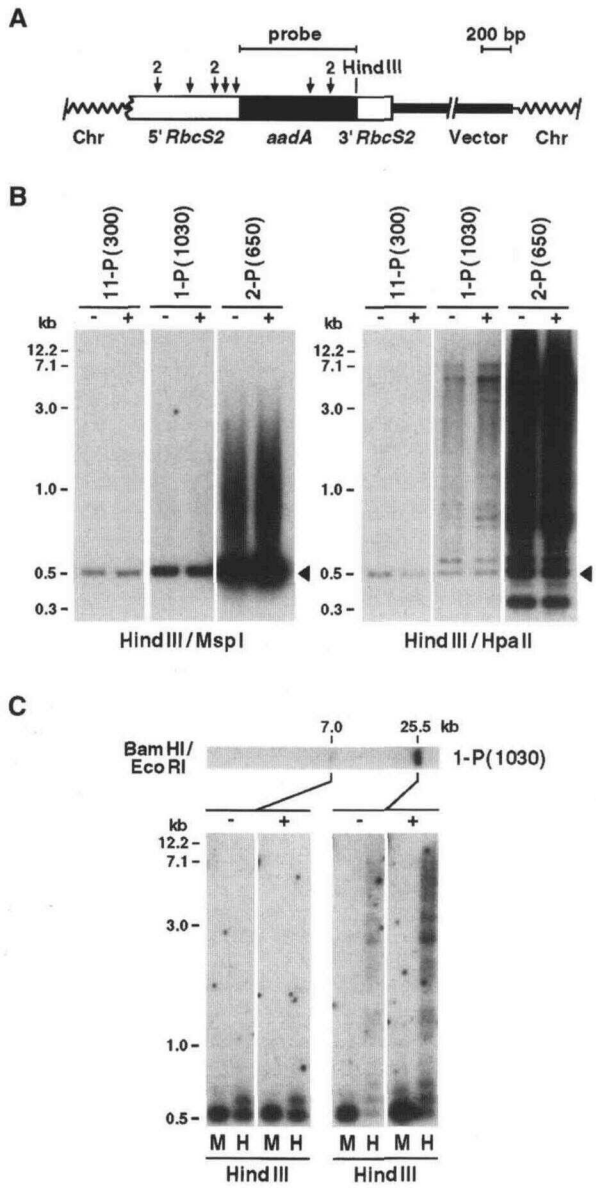


Figure 6. Methylation of Cytosine Residues Does Not Correlate with Transcriptional Silencing of the Introduced *RbcS2*::*aadA*::*RbcS2* Gene(s).

(A) Diagram of one copy of plasmid DNA integrated into the Chlamydomonas nuclear genome. The arrows indicate the positions of MspI-HpaII restriction sites. A number 2 above an arrow indicates two very close sites. Chlamydomonas chromosomal (Chr) DNA is shown as wavy lines.

(B) Gel blots of total DNA isolated from the indicated transformants maintained under nonselective (−) or selective (+) conditions. Total cell DNA was digested with HindIII and MspI or HindIII and HpaII and probed with the coding sequence of *aadA*. The arrowheads indicate the 530-bp fragment corresponding to unmethylated DNA (see text for details). Numbers at left indicate the positions of molecular weight DNA markers in kilobases.

RbcS2 promoter region of the introduced gene(s) by digestion with BstEII and MspI or BstEII and HpaII and probing with a segment encompassing the 5′ end of the *aadA* coding sequence (Figure 7A). In the absence of cytosine methylation, a fragment of 230 bp is observed (Figure 7A). The radioactivity detected in this 230-bp band was used to estimate the number of unmethylated copies in subclones maintained under selective or nonselective conditions, as described in Methods. As before, subclones from the same transformant showed the same degree of methylation, independent of the selective conditions (Table 3; data not shown). Similar results were obtained by evaluating methylation at the SmaI-XmaI site (Figure 7A) in the *RbcS2* upstream sequence (data not shown).

Our analysis determined the average number of copies that are not methylated at two specific sites in the promoter region of the integrated *RbcS2*::*aadA*::*RbcS2* genes in multiple-copy transformants (Table 3). However, these results might be due to lack of methylation along the whole sequence in certain copies and complete methylation in others (i.e., all-or-none methylation states) or to different extents of methylation at different sites in all copies (i.e., mixed methylation states). In the case of transformant 1-P(1030), we could distinguish between these possibilities because the ~5.5 copies of the *RbcS2*::*aadA*::*RbcS2* gene integrated at two closely linked sites (Cerutti et al., 1997). An array of ~4.5 copies could be separated as a fragment of 25.5 kb after digestion with BamHI and EcoRI, whereas the remaining copy migrated as a 7-kb segment (Figure 6C; Cerutti et al., 1997). BamHI- and EcoRI-digested genomic DNA from transformant 1-P(1030) was fractionated by agarose gel electrophoresis, and DNA in broad bands corresponding to fragments of 20 to 30 and 6 to 8 kb was purified from the gel. The methylation status of the isolated DNA was evaluated by digestion with HindIII and MspI or HindIII and HpaII, as described above.

The ~4.5 copies in the array displayed heavy methylation, which was manifested as a ladder of high molecular weight fragments in the HpaII-digested lanes (Figure 6C). In contrast, the single-copy insert was only slightly methylated (Figure 6C). These results were confirmed with another methylation-sensitive enzyme (HhaI; data not shown). Thus, our observations suggest that in multiple-copy transformants, some of

(C) The ~5.5 copies of the *RbcS2*::*aadA*::*RbcS2* gene have integrated at two closely linked sites in transformant 1-P(1030) and are revealed as two *aadA*-hybridizing bands on gel blots of BamHI- and EcoRI-digested total DNA (see text for details). These fragments from subclones propagated in the absence (−) or presence (+) of spectinomycin were purified from agarose gels and analyzed for methylation of cytosine residues, as described in **(B)**. Numbers at left indicate the positions of molecular weight DNA markers in kilobases. H, HpaII; M, MspI.

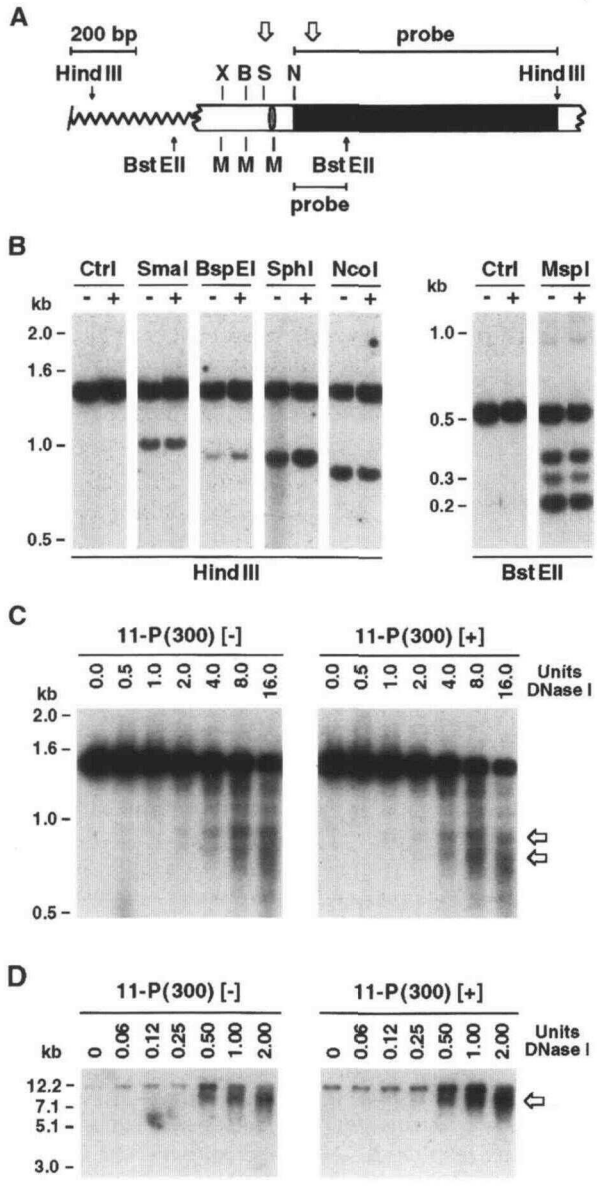


Figure 7. The Chromatin Structure of the *RbcS2*::*aadA*::*RbcS2* Gene in Transformant 11-P(300) Is Very Similar in Subclones Differing >10-Fold in Transcriptional Activity.

(A) Diagram of the single *RbcS2*::*aadA*::*RbcS2* gene integrated in transformant 11-P(300). The ellipse in the *RbcS2* upstream sequence indicates the location of the TATA element. The open arrows show DNase I hypersensitive sites. Restriction sites: B, BspEI; M, MspI; N, NcoI; S, SphI; X, XmaI (SmaI).

(B) Gel blots of total DNA demonstrating the accessibility to restriction enzymes of the introduced transgene in subclones of transformant 11-P(300) maintained under nonselective (−) or selective (+) conditions. Liquid nitrogen–permeabilized cells were treated with the indicated restriction enzymes, as described in Methods. Purified total cell DNA was digested with HindIII and probed with the coding sequence of *aadA*. In the case of MspI-treated cells, genomic DNA was digested with BstEII and probed with the NcoI-BstEII fragment corresponding to the 5′ end of the *aadA* coding sequence. Numbers at left indicate the positions of molecular weight DNA markers in kilobases. Ctrl, liquid nitrogen–permeabilized cells incubated in the absence of restriction endonucleases.

(C) Gel blots of total DNA demonstrating the accessibility to DNase I of the introduced transgene in subclones of transformant 11-P(300) maintained under nonselective (−) or selective (+) conditions. Liquid nitrogen–permeabilized cells were incubated with increasing amounts of DNase I, as described in Methods. Purified total cell DNA was digested with HindIII and probed with the *aadA* coding sequence. The open arrows indicate the fragments generated by digestion at DNase I hypersensitive sites. Numbers at left indicate the positions of molecular weight DNA markers in kilobases.

(D) Samples treated as in **(C)** were probed with a 550-bp fragment of the *RbcS2* promoter region immediately upstream of the sequence included in construct P(300) (Cerutti et al., 1997). A strong DNase I hypersensitive site (open arrow) was detected, suggesting that the native chromatin structure is preserved in permeabilized cells. Numbers at left indicate the positions of molecular weight DNA markers in kilobases.

the integrated copies are almost completely devoid of methylation. As before, we did not detect any obvious difference in the methylation status of transformant 1-P(1030) maintained under selective or nonselective conditions (Figure 6C; cf. subclones designated [−] and [+]). Therefore, cytosine methylation does not appear to be involved in the transcriptional inactivation of the introduced *RbcS2*::*aadA*::*RbcS2* gene(s) in single-copy transformants and in some copies of multiple-copy transformants. However, in the latter, we cannot exclude the possibility that extensive cytosine methylation may inhibit transcription of some of the integrated *RbcS2*::*aadA*::*RbcS2* genes.

Transcriptional Inactivation of the *RbcS2*::*aadA*::*RbcS2* Gene(s) Does Not Correlate with Large Alterations in the Accessibility of the Chromatin-Packaged DNA to Restriction Endonucleases and DNase I

In Drosophila, euchromatic genes subject to PEV are frequently responsive to temperature. At low temperatures, there is greater repression of gene expression (i.e., enhancement of variegation) (Spofford, 1976; Michailidis et al., 1988). A similar effect has been observed for the mosaic expression of the *ura4* and *ade6* genes inserted at centromeres in *S. pombe* (Allshire et al., 1994). Because gene silencing in these organisms appears to be chromatin mediated, we examined whether expression of the *RbcS2*::*aadA*::*RbcS2* gene(s) in Chlamydomonas transformants was also sensitive

to temperature, perhaps providing an indication of chromatin involvement in the observed transcriptional inactivation.

Cells of transformants grown to middle log phase at 25°C were serially diluted, spotted onto TAP plates or spectinomycin-containing TAP plates, and incubated at 25 or 17°C. All subclones maintained under nonselective conditions grew on the selective plates at 25°C, although to different extents as reflected by the intensity, number, and size of the colonies in serially diluted spots (Figure 8A). In contrast, the growth of these subclones under selective conditions was substantially reduced at 17°C (Figure 8A). Similar temperature effects were observed with the subclones maintained constantly under selective conditions, although the reduction in growth at 17°C on plates containing spectinomycin was less pronounced (Figure 8B). This effect of temperature on transgene expression appears to be specific for Chlamydomonas transformants showing an unstable Spc^r phenotype, because stable nuclear transformants or a chloroplast transformant expressing *aadA* under the control of the *atpA* promoter did not exhibit growth differences on selective plates at 25 or 17°C (Figure 8, Chl Trans; data not shown). Thus, our observations suggest that lower temperatures reduce expression of the integrated *RbcS2*::*aadA*::*RbcS2* gene(s) in Chlamydomonas transformants subjected to epigenetic gene silencing.

By analogy to Drosophila and fission yeast, these results suggest that expression of the *RbcS2*::*aadA*::*RbcS2* gene(s) in Chlamydomonas might be influenced by chromatin structure. To determine whether reduced transcriptional activity was associated with altered chromatin packaging, we examined the accessibility of DNA to restriction endonucleases or DNase I in isolated nuclei and permeabilized cells. However, methylated DNA is frequently accompanied by a condensed chromatin structure poorly accessible to enzymatic probes (Keshet et al., 1986; Eden and Cedar, 1994; Ye and Signer, 1996; but see also Weng et al., 1995). Thus, discriminating the possible effects of cytosine methylation from those of chromatin packaging in methylated multiple-copy transformants would be difficult. Therefore, we examined two single-copy transformants, 11-P(300) and 27-P(1030), subjected to pronounced gene silencing (Tables 1 and 3) but lacking any detectable methylation of the integrated *RbcS2*::*aadA*::*RbcS2* gene (see above). As an example, the analysis of transformant 11-P(300) is shown in Figure 7, but similar results were obtained with transformant 27-P(1030) (data not shown).

Liquid nitrogen–permeabilized cells (Gagné and Guertin, 1992) from subclones kept under selective or nonselective conditions were incubated with an excess amount of the restriction enzymes NcoI, MspI, SphI, BspEI, or SmaI, which cleave immediately downstream of the translation start codon (NcoI) or at several sites in the promoter sequence of the *RbcS2*::*aadA*::*RbcS2* gene (Figure 7A). Genomic DNA was purified and digested to completion with BstEII, in the case of MspI-treated cells, or with HindIII, in the case of cells incubated with other restriction enzymes (Figure 7A). The fre-

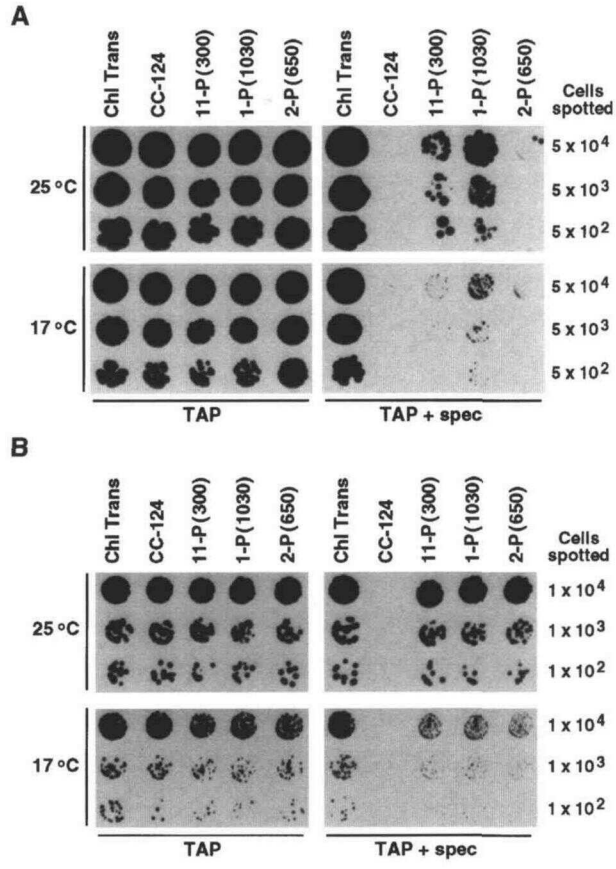


Figure 8. Effect of Low Temperature on the Spectinomycin Resistance of Chlamydomonas Transformants with an Unstable Spc^r Phenotype.

The plates show the growth and survival at 25 or 17°C, in the absence (TAP) or presence (TAP + spec) of spectinomycin, of the indicated transformants, the wild-type strain CC-124, and a chloroplast transformant (Chl Trans) expressing *aadA* under the control of the *atpA* promoter (Goldschmidt-Clermont, 1991). Cells grown mixotrophically to middle log phase were serially diluted in TAP medium to the indicated numbers, spotted on the plates, and incubated at the indicated temperatures. Plates at 17°C were incubated for longer times, until the growth of the cells on the nonselective plates was approximately equivalent to that at 25°C.

(A) Subclones of unstable spectinomycin-resistant Chlamydomonas transformants maintained under nonselective conditions. The plates show the growth of the cells after 10 or 21 days of incubation at 25 or 17°C, respectively.

(B) Subclones of unstable spectinomycin-resistant Chlamydomonas transformants kept under selective conditions. The plates show the growth of the cells after 7 or 14 days of incubation at 25 or 17°C, respectively.

quency of cleavage at the different sites was determined by DNA gel blot hybridization using as probes the NcoI-BstEII fragment at the 5′ end of the *aadA* coding sequence or the complete *aadA* coding sequence (Figure 7A). There was no significant difference in the accessibility of any of the restriction sites tested between subclones maintained under selective or nonselective conditions (Figure 7B; cf. subclones designated [−] and [+]). In both cases, the greatest cleavage (35 to 45%) corresponded to the SphI and the MspI sites flanking the TATA element (Figures 7A and 7B).

Because digestion with restriction enzymes can only evaluate a limited number of sites, we also incubated isolated nuclei or permeabilized cells with increasing amounts of DNase I (see Methods). The DNA was purified and digested to completion with HindIII. DNA gel blot analysis with the complete *aadA* coding sequence as probe (Figure 7A) revealed two predominant, although rather weak, hypersensitive sites (Figure 7C). Controls with naked DNA did not show appreciable sequence bias in the cleavage of this region by DNase I (data not shown). One DNase I–hypersensitive site corresponded to the region around the TATA box (Figure 7A) that was also more accessible to restriction enzyme digestion (Figure 7B). The other DNase I–hypersensitive site was located at the 5′ end of the *aadA* coding sequence, close to the translation start codon (Figure 7A). However, in agreement with the restriction endonuclease analyses, we observed no significant differences in the pattern of hypersensitive sites or in overall sensitivity to DNase I between subclones maintained in the presence or absence of spectinomycin (Figure 7C; cf. 11-P(300) [−] and 11-P(300) [+]), even after extensive digestion. As a control for intactness of the chromatin structure in isolated nuclei or permeabilized cells, we also determined the sensitivity of the endogenous *RbcS2* gene to digestion with DNase I. A strong hypersensitive site was detected in all subclones (Figure 7D), a few kilobases upstream of the *RbcS2* transcription start. This site was not included in our constructs containing ≤1 kb of promoter sequence (Cerutti et al., 1997).

In summary, our results reveal that the chromatin organization of the integrated *RbcS2*::*aadA*::*RbcS2* gene, by low-resolution analysis (Lu et al., 1993), is very similar in subclones differing in their transcriptional activity by more than one order of magnitude. These observations do not seem to reflect a lack of preservation of the native chromatin structure in our samples, because both the endogenous *RbcS2* gene and the introduced chimeric gene showed differential accessibility of several sites to enzymatic probes, consistent with them being assembled into organized chromatin domains. Moreover, the same results were obtained with freshly isolated nuclei as well as with cells permeabilized by freezing in liquid nitrogen or by treatment with nystatin (see Methods). The latter procedure has been demonstrated to be particularly successful at avoiding disturbance of chromatin organization in *S. cerevisiae* (Venditti and Camilloni, 1994; Verdone et al., 1996). We conclude therefore that transcriptional silencing of the introduced *RbcS2*::*aadA*::*RbcS2* gene in single-copy transformants of Chlamydomonas does not appear to correlate with gross changes in chromatin packaging that would limit access of the DNA to relatively small proteins, such as restriction endonucleases or DNase I. These results are not compatible with transcriptional repression being caused by a compacted chromatin structure capable of blocking all interactions between DNA binding proteins and their DNA sites.

DISCUSSION

A long-standing problem in the engineering of the Chlamydomonas nuclear genome has been the inability to express introduced foreign gene sequences (Blankenship and Kindle, 1992; Stevens et al., 1996; Cerutti et al., 1997). The actual reasons for this failure have not been analyzed in any detail, although our recent studies suggest that several independent problems, such as gene silencing and mRNA instability, might be involved (Cerutti et al., 1997). In Chlamydomonas, both homologous and heterologous sequences integrated randomly into the nuclear genome undergo cytosine methylation (Blankenship and Kindle, 1992; Ferris, 1995), but the role of this DNA modification in gene expression/repression remains unknown. To begin to understand these phenomena, we examined the mechanism(s) responsible for the inactivation of foreign gene sequences in the nuclear genome of Chlamydomonas and how it relates to epigenetic gene silencing in other eukaryotic organisms.

Chlamydomonas nuclear transformants expressing the eubacterial aminoglycoside 3′-adenyltransferase (*aadA*) gene under the control of the regulatory sequences of the Chlamydomonas *RbcS2* gene (Cerutti et al., 1997) frequently showed an unstable Spc^r phenotype. Cosegregation of the Spc^r phenotype with the introduced *RbcS2*::*aadA*::*RbcS2* gene(s) in the progeny of sexual crosses confirmed that antibiotic resistance was being conferred by expression of this chimeric gene(s). Clonal pedigree analysis of representative transformants indicated that (1) genetically identical cells can revert to the opposite phenotype (i.e., from Spc^r to Spc^s and vice versa) at frequencies significantly higher than expected for spontaneous mutation; and (2) these phenotypic changes do not involve loss or large deletions/rearrangements of the integrated transforming DNA. Taken together, these results suggested that expression of the *RbcS2*::*aadA*::*RbcS2* transgene(s) was being repressed by an epigenetic silencing mechanism(s). Moreover, nuclear run-on analyses clearly demonstrated reduced transcriptional activity of the chimeric gene(s) in subclones maintained under nonselective conditions.

Although our results indicate that the introduced *RbcS2*::*aadA*::*RbcS2* gene(s) is silenced at the transcriptional level, several lines of evidence suggest that different levels of expression/repression can be established and maintained in genetically identical cells. Under selective conditions, colonies of a given unstable spectinomycin-resistant transformant

(ultimately derived from a single transformed cell) showed great heterogeneity in size consistent with multiple intermediate degrees of expression of the introduced selectable marker, and therefore spectinomycin resistance, among progeny cells. Moreover, subclones derived from individual cells of a given transformant showed different extents of survival on spectinomycin, even when propagated under selective conditions for 12 weeks. In plating experiments, increasing numbers of surviving cells were observed as the concentration of spectinomycin on the selective plates was reduced, although the lowest concentration of antibiotic was still lethal for wild-type Chlamydomonas. Because transcription of the introduced chimeric gene(s) correlates with the steady state levels of the corresponding mRNA and protein (this work; Cerutti et al., 1997), the resistance to spectinomycin of individual cells most likely reflects their transcriptional activity. However, relative transcription, estimated by nuclear run-on assays, was significantly higher than relative cell survival on spectinomycin. This supports the existence of intermediate expression states (below the threshold required for survival on 75 μg/mL spectinomycin) rather than a simple switch between two alternate, on/off states (see Results for details).

These multiple expression/repression states appear to be mitotically metastable for at least 15 to 20 generations, based on the predominantly well-rounded shape of individual colonies and direct measurements of reversion to spectinomycin resistance. However, the intermediate states are much less stable meiotically. In fact, the inactive state(s), characterized by an expression level below the threshold required for survival under our selective conditions, seems to be the most stable both mitotically and meiotically, and most cells eventually become silenced. A high level of quantitative variation with multiple metastable intermediate phenotypes has also been observed in paramutation of the maize *pl* and *r* loci (which, like *b*, encode transcriptional activators of the anthocyanin biosynthetic pathway) (Brink, 1973; Hollick et al., 1995; Kermicle et al., 1995; Patterson and Chandler, 1995). Moreover, a trend toward reduced gene activity also appears to be favored in at least some cases of maize paramutation, where the weakened paramutant derivatives are very stable (Hollick et al., 1995; Kermicle et al., 1995; Patterson and Chandler, 1995; Patterson et al., 1995).

Patterns of cellular mosaicism of transgene expression were also observed in higher plants subject to epigenetic silencing phenomena (Neuhuber et al., 1994; Jorgensen, 1995). However, in cases where transcriptional repression has been detected, it is not always possible to assess whether reduced transcription reflects a uniform decrease in all cells or an intermediate phenotype resulting from complete inactivation in some cells and normal transcriptional activity in others (Matzke and Matzke, 1995). In contrast, our results strongly suggest that different metastable transcriptional states can be established in genetically identical cells. Similarly, intermediate levels of expression in individual cells have been reported for genes exhibiting variegation because of their integration at centromeres in *S. pombe* (Allshire et al., 1994).

Possible Mechanism(s) of Transcriptional Gene Silencing in Chlamydomonas

Multiple-copy transformants have some copies of the integrated *RbcS2*::*aadA*::*RbcS2* genes that seem to lack methylation. Because these transformants were isolated by direct selection on spectinomycin-containing TAP plates, they must be able to express one or more copies of the introduced *RbcS2*::*aadA*::*RbcS2* gene(s). We speculate that this copy(ies) is not methylated and remains as such. Indeed, the transcriptional activity in subclones maintained under selection seems to correlate with the estimated number of unmethylated copies (Table 3). However, this also suggests that extensively methylated DNA is completely repressed and that cytosine methylation might play a role in the silencing of repeated transgenes in Chlamydomonas (see also Blankenship and Kindle, 1992). In fact, DNA methylation has been shown to correlate with gene inactivation in several eukaryotes (Eden and Cedar, 1994; Rossignol and Faugeron, 1994; Martienssen and Richards, 1995; Singer and Selker, 1995; Weng et al., 1995; Hohn et al., 1996; Meyer and Saedler, 1996), although the causal relationship between this DNA modification and gene expression is not clear in many instances.

Silenced single-copy transformants of Chlamydomonas do not display detectable methylation of the introduced chimeric gene, and as already mentioned, some copies of silenced multiple-copy transformants do not appear to be methylated. With the caveat that subtle changes in methylation, particularly at nonsymmetric sites, would have gone undetected in our analysis using methylation-sensitive restriction enzymes, our results suggest the existence of an additional mechanism of transcriptional inactivation independent of methylation. Titration of essential transcription factors by the introduced transgene(s) seems unlikely, because expression of the endogenous *RbcS2* gene, having the same regulatory sequences, is not affected (this work; Cerutti et al., 1997).

Transcriptional repression in several eukaryotes can also result from DNA pairing interactions in *trans* (*trans*-sensing effects), typically between allelic genes on homologous chromosomes (Brink, 1973; Rossignol and Faugeron, 1994; Henikoff, 1995; Matzke and Matzke, 1995; Patterson and Chandler, 1995; Singer-and Selker, 1995; Aramayo and Metzenberg, 1996). *Trans*-sensing effects underlie a number of complex epigenetic phenomena, several of which do not involve cytosine methylation of the DNA, such as transvection in Drosophila (Chen and Pirrota, 1993; Henikoff, 1995) and paramutation at the *b* locus in maize (Patterson and Chandler, 1995; Patterson et al., 1995). In these examples, repression depends on the continuous interaction of susceptible alleles (transvection), or the less active allele heritably imposes its condition on the sensitive, more active allele (paramutation).

Similar *trans*-sensing effects cannot explain the silencing of single copies of the introduced *RbcS2*::*aadA*::*RbcS2* gene in haploid Chlamydomonas cells, because the only homologous sequences that potentially could interact correspond to the regulatory regions of *RbcS2*. This would also affect expression of the endogenous gene, something we have not observed because the rate of transcription and the steady state mRNA level of the *RbcS2* gene are not reduced in silenced transformants (this work; Cerutti et al., 1997).

Based on ultrastructure, nucleosomal spacing, and the complement of histones, Chlamydomonas nuclear chromatin is very similar to that of both mammals and higher plants (Morris et al., 1990). However, there appear to be no data on the presence or distribution of heterochromatin in the chromosomes of this alga determined by cytological methods. Nonetheless, our results on epigenetic silencing of the introduced *RbcS2*::*aadA*::*RbcS2* gene(s) suggest several parallels with (hetero)chromatic gene repression in other species. The survival on spectinomycin of Chlamydomonas transformants was sensitive to temperature, with lower temperatures reducing the survival and presumably the level of chimeric gene expression. (Hetero)chromatin-mediated variegated gene expression in Drosophila and fission yeast is similarly affected by temperature (Spofford, 1976; Michailidis et al., 1988; Allshire et al., 1994). As mentioned above, intermediate levels of expression in individual cells, resembling our observations in Chlamydomonas, have been reported for transgenes inserted at centromeres in *S. pombe* (Allshire et al., 1994). Similarly, we have found that the integrated transforming DNA is centromere linked in Chlamydomonas transformant 1-P(1030) (data not shown) and thus could be associated with pericentromeric heterochromatin. We are currently mapping the insertion loci in transformants 11-P(300) and 2-P(650) to determine whether they are also located near centromeres. In addition, pairing between repeated sequences in multiple-copy transformants might induce local heterochromatin formation, as reported for euchromatically located repeat arrays of a P element in Drosophila (Dorer and Henikoff, 1994; Sabl and Henikoff, 1996).

In several organisms, chromatin-mediated gene silencing is frequently associated with reduced accessibility of the DNA in nuclei to enzymatic probes, such as DNase I, restriction endonucleases, or DNA methyltransferases (Gottschling, 1992; Allshire et al., 1994; Loo and Rine, 1994; Wallrath and Elgin, 1995; Festenstein et al., 1996; Milot et al., 1996). However, several genes subject to PEV in Drosophila show no change or only a slight reduction in chromatin accessibility to enzymatic probes (Hayashi et al., 1990; Locke, 1993; Schlossherr et al., 1994; Wines et al., 1996). Moreover, Milot et al. (1996) have recently demonstrated that β-globin transgenes with an incomplete locus control region show variegated expression in mice when integrated into heterochromatic, centromere-associated regions. Two different silencing mechanisms were apparently responsible for these observations. One was consistent with the on/off transcriptional states of classical PEV and appeared to involve a condensed chromatin conformation less accessible to DNase I and restriction enzymes. The other, in contrast, did not involve changes in chromatin accessibility and apparently resulted from a decrease in the period of time during the cell cycle that the transgene is transcriptionally active in all cells (Milot et al., 1996). In another parallel with some cases of chromatin-mediated gene silencing, we have not detected significant changes in chromatin organization, examined by digestion with restriction endonucleases and DNase I, between Chlamydomonas subclones differing by >10-fold in transcriptional activity.

Transcriptional repression of the integrated *RbcS2*::*aadA*::*RbcS2* gene(s) in Chlamydomonas transformants might occur via a variety of different molecular mechanisms, such as those proposed to explain some cases of chromatin-mediated gene silencing (reviewed in Elgin, 1996; Felsenfeld, 1996; McCall and Bender, 1996; Sabl and Henikoff, 1996). A number of recent studies have suggested that the location of genes in chromosomal domains within the interphase nucleus is important for normal expression (Weiler and Wakimoto, 1995; Csink and Henikoff, 1996; Dernburg et al., 1996; Sabl and Henikoff, 1996). For instance, there is evidence in Drosophila that heterochromatin and euchromatin are functionally different compartments in terms of gene expression. When a gene is moved from its normal location in one domain to a site within the other domain, it is often subject to mosaic inactivation (Dorer and Henikoff, 1994; Karpen, 1994; Henikoff, 1995; Weiler and Wakimoto, 1995; Sabl and Henikoff, 1996). Moreover, long-range interactions between centromeric heterochromatin and other regions of heterochromatin on the same chromosome can modulate the position of genes within the interphase nucleus and determine their transcriptional activity (Csink and Henikoff, 1996; Dernburg et al., 1996). In *S. cerevisiae*, there is also evidence indicating that nuclear localization plays a role in gene silencing (Maillet et al., 1996).

In Chlamydomonas, as proposed for β-globin transgenes in mice (Milot et al., 1996), transgenes might be organized into chromosomal domains that persist throughout the cell cycle and determine the extent and timing of accessibility of the introduced genes to the transcription machinery. A transgene packaged into a fully heterochromatic domain might only be accessible for transcription during late S phase, perhaps coincidental with the chromatin reorganization required for DNA replication (see also Riggs and Pfeifer, 1992). Packaging of the transgenes into distinct chromosomal domains, allowing transcription for different periods of the cell cycle, could explain the various levels of expression in genetically identical cells and account for the mitotic metastability of these states. Furthermore, pairing interactions between homologs at meiosis could disrupt chromosomal organization, allowing the establishment of new, presumably more repressive, domains. Experiments designed to test these hypotheses are currently under way.

A similar mechanism(s) of transcriptional gene silencing might also operate in higher plants. Some transgenic loci that are silenced and act as strong transcriptional silencers

are known to be located close to telomeres, presumably associated with heterochromatin, although a role for methylation in these cases of gene inactivation cannot be ruled out (Matzke et al., 1994; Neuhuber et al., 1994; Elmayan and Vaucheret, 1996; Park et al., 1996). Both silencing of *cab140* in transgenic Arabidopsis (Brusslan et al., 1993) and paramutation at the *b* locus in maize (Patterson and Chandler, 1995; Patterson et al., 1995) involve epigenetic transcriptional inactivation that does not correlate with methylation. Moreover, in the sense suppression of chalcone synthase in petunia, a complicated hierarchy of transcriptional and post-transcriptional mechanisms has been proposed to cause the diversity, complexity, and metastability of the multiple coloration patterns frequently observed in flowers (Jorgensen, 1995; Jorgensen et al., 1996). Here, we provide strong evidence that multiple expression states of introduced genes in Chlamydomonas can indeed be established at the transcriptional level in the clonal progeny of individual cells. Furthermore, these states can be modified by environmental factors such as temperature.

In summary, our results demonstrate the existence of epigenetic transcriptional inactivation of a foreign gene in the nuclear genome of the unicellular green alga Chlamydomonas. Although methylation might be involved in the silencing of certain copies of repeated transgenes, an additional mechanism of inactivation appears to be operative. In higher plants, transcriptional inactivation of transgenes has been proposed to result from DNA methylation and/or a condensed chromatin structure, perhaps triggered in some cases by DNA–DNA or DNA–RNA interactions (Matzke and Matzke, 1995; Ten Lohuis et al., 1995; Meyer and Saedler, 1996; Park et al., 1996; Ye and Signer, 1996). In Chlamydomonas, the multiple expression states, temperature sensitivity of expression, chromosomal location, and lack of methylation or altered chromatin accessibility of the introduced *RbcS2*::*aadA*::*RbcS2* gene(s) all suggest a possible role for (hetero)chromatic chromosomal domains in this epigenetic phenomenon.

METHODS

Strains, Culture Conditions, and Genetic Analysis

Wild-type *Chlamydomonas reinhardtii* CC-124 (*mt*$^-$) was used for all transformation experiments. Unless noted otherwise, cells were grown mixotrophically at 25°C in Tris–acetate–phosphate (TAP) medium (Harris, 1989), as previously described (Cerutti et al., 1995, 1997). Cell survival on spectinomycin was examined by spreading the cells on TAP–agar plates or TAP–agar plates containing 75 μg/mL spectinomycin. For each transformant, five replicates of 500 to 700 cells per plate were incubated under moderate light (110 μmol m^{-2} sec^{-1} photosynthetically active radiation) at 25°C for 10 days before the colonies were counted. The percentage of plated cells that survived and formed colonies on spectinomycin was determined relative to the number of colonies on the nonselective plates to correct for differences in plating efficiency between transformants. For spot tests, 5-μL aliquots of appropriately diluted cells (see the legend to Figure 8) were pipetted onto the plates and incubated at 25 or 17°C, as indicated. Subclones of transformants were maintained as streaks on TAP–agar plates (nonselective conditions) or on TAP–agar plates containing 75 μg/mL spectinomycin (selective conditions). Cells were streaked on fresh plates every 2 weeks. Mating of the transformants to the wild-type strain CC-125 (*mt*$^+$) and tetrad analysis were performed as described by Harris (1989). High-efficiency-mating strains CC-620 and CC-621 were used to prepare gamete autolysin (Harris, 1989).

Transformation of Chlamydomonas with the Chimeric *RbcS2*::*aadA*::*RbcS2* Genes

The constructs and the procedures used for nuclear transformation of Chlamydomonas have been described previously (Cerutti et al., 1997).

DNA and RNA Analyses of Chlamydomonas Transformants

Nucleic acids were isolated from Chlamydomonas transformants as previously described (Cerutti et al., 1995, 1997). Standard techniques were used for DNA and RNA gel blot analyses of these transformants (Sambrook et al., 1989; Cerutti et al., 1992, 1995, 1997). To determine the number of copies of the *RbcS2*::*aadA*::*RbcS2* gene integrated into the nuclear genome, total cell DNA was digested with NcoI and HindIII to excise the *aadA* coding sequence (Figure 7A). Digested DNA was fractionated by agarose gel electrophoresis, blotted to nylon membranes, and probed with the radioactively labeled *aadA* sequence. Total radioactivity in the lanes was quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and then compared with the radioactivity corresponding to a single-copy transformant. Differences in loading were corrected by normalizing to the amount of chloroplast DNA, detected by hybridization with the *chlL* probe (Cerutti et al., 1995). The number of unmethylated copies was estimated by gel blot analysis of total DNA digested with BstEII and HpaII and probed with the NcoI-BstEII segment encompassing the 5′ end of the *aadA* coding sequence (Figure 7A). In the absence of cytosine methylation, a fragment of 230 bp was observed resulting from cleavage at the HpaII-MspI site in the promoter region, immediately downstream of the TATA element, and at the methylation-insensitive BstEII site inside the *aadA* coding sequence (Figure 7A). The radioactivity detected in the expected 230-bp fragment was compared with the radioactivity in this band for an unmethylated single-copy transformant. As before, differences in loading were corrected by normalizing to the amount of chloroplast DNA. When needed, DNA fragments were purified from agarose gels by electroelution (Sambrook et al., 1989).

Nuclear Run-On Transcription Assays

Intact nuclei were isolated and used to determine transcriptional activity by a modification of published procedures (Keller et al., 1984; Dehio and Schell, 1994), including pretreatment of the cells with gamete autolysin to remove the cell walls. Nuclear transcripts, labeled with ^{32}P-rUTP, were used as probes on filters containing an excess of target DNA, and the radioactivity retained on the slots was quantified with a PhosphorImager, as previously described (Cerutti et al., 1997).

Analysis of Chromatin Accessibility to Enzymatic Probes

Cells of Chlamydomonas transformants were grown mixotrophically to middle log phase, pelleted, and washed twice with buffer I (10 mM

Tris-HCl, pH 8.0, 0.5 mM EDTA, 50 mM KCl, 250 mM sucrose, and 1 mM DTT). The pellet of cells was resuspended in the same buffer to a density of 10^8 cells per mL, and 500-μL aliquots were frozen in liquid nitrogen, as described by Gagné and Guertin (1992). Permeabilized cells were allowed to thaw on ice and washed once with buffer II (25 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl, 10% glycerol, and 0.2 mM DTT) at 4°C. Aliquots of ~2×10^7 cells in 100 μL of buffer II were treated with increasing concentrations of DNase I (Boehringer Mannheim), as indicated in Figures 7C and 7D, at 4°C for 10 min. The reactions were stopped, and DNA was purified as previously described for nuclear run-on assays (Cerutti et al., 1997). For restriction endonuclease treatment, liquid nitrogen–permeabilized cells were washed once with buffer II and resuspended in the manufacturer's recommended buffer (containing 5 μg/mL leupeptin and 0.15 units per mL of aprotinin) to a density of ~2.5×10^8 cells per mL. Aliquots of 200 μL of cells were treated with 200 units of restriction endonuclease at 22°C for 30 min. The reactions were stopped, and DNA was purified as before. Total cell DNA digested with HindIII or BstEII was analyzed by gel blotting according to standard procedures (Sambrook et al., 1989; Cerutti et al., 1992, 1995).

Freshly isolated nuclei (Cerutti et al., 1997) or nystatin-permeabilized cells were also used for DNase I treatments. For nystatin permeabilization, cells were grown as before and incubated for 30 to 60 min with gamete autolysin to remove the cell walls (Harris, 1989). Protoplasts were subsequently washed twice with buffer I and twice with buffer II at 4°C. Finally, the protoplast pellet was resuspended to a density of ~2×10^8 cells per mL in buffer II containing 100 μg/mL nystatin (Venditti and Camilloni, 1994). Aliquots of 100 μL of resuspended protoplasts were treated with increasing concentrations of DNase I at 4°C for 15 min, and the DNA was purified as before. Preliminary experiments were performed to ensure that freezing in liquid nitrogen or incubation in the presence of nystatin permeabilized >99% of the cells (data not shown).

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