A New Assay for Promoter Analysis in *Chlamydomonas* Reveals Roles for Heat Shock Elements and the TATA Box in *HSP70A* Promoter-Mediated Activation of Transgene Expression

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The aim of this work was to identify cis-regulatory sequences within the *Chlamydomonas HSP70A* promoter that mediate its stimulatory effect on the expression of downstream promoters. For this, we deleted/mutated the *HSP70A* promoter and, using a new assay, quantified its stimulatory effect. Our results indicate that the effect is mediated largely by heat shock elements and the TATA box.

Transgenic approaches with *Chlamydomonas* often suffer from the low percentage of transformants that express a stably integrated transgene (e.g., 4, 5, 13). We found that transgene expression in *Chlamydomonas* was significantly improved when the *Chlamydomonas* *HSP70A* (*A*) promoter was fused upstream from the transgene-driving promoter (12, 13, 14). The *A* promoter apparently acted as a transcriptional state enhancer; i.e., it improved the probability of randomly integrated transgenes becoming expressed (10, 14). Enhancing activities, however, could be assigned to two different regions of the *A* promoter, a proximal region (ranging from bp −23 to −285 upstream from the translational start codon) and a distal region (upstream from bp −286).

The goal of this work was to identify cis-regulatory sequences within the *A* promoter by means of which it mediates activation of transgene expression. To this end, *A* promoter deletions and mutations were fused upstream from the *RBCS2* promoter (*R*) to drive expression of the *ble* gene, conferring resistance to zeocin (9). In earlier work, we estimated the activation efficiency of *A* promoter derivatives (i) by counting zeocin-resistant colonies produced by cells directly transformed with *R-ble/AR-ble* constructs and (ii) by determining the fraction of *ble*-expressing, zeocin-resistant arginine-prototrophic transformants that emerged from arginine-auxotrophic cells cotransformed with the *ARG7* gene and *R-ble/AR-ble* constructs (14). As these methods were tedious and/or led to statistically insignificant results, we sought for an alternative assay to quantify the activating effect of the *A* promoter. We reasoned that it might be possible to quantify the amount of transgene transcript produced per intact transgene in pools of hundreds of cotransformants generated with the *ARG7* gene and *R-ble/AR-ble* constructs. As in our hands around 20 to 60% of cotransformants contain the cotransformed construct and in case of *R-ble* ~20% of the transgenes are expressed (14), the *R-ble* construct was expected to be expressed in only 4 to 12% of the cotransformants. To test whether Northern analysis was sensitive enough to detect such low *ble* mRNA levels in pools of cotransformants, a liquid culture in TAP medium (6) of a *Chlamydomonas* strain containing an expressing *AR-ble* construct was increasingly diluted with a culture of a strain containing a nonexpressing *R-ble* construct (all transformants were generated with strain cw15-302, kindly provided by R. Matagne, University of Liège, Belgium). Northern analysis of RNA extracted from these cells (as described in reference 7) revealed that *ble* transcript was detectable when only 1% of the cells expressed a *ble* construct and that *ble* signals correlated well with the fraction of expressing cells (Fig. 1A).

To quantify the number of intact transgenes in pools of cotransformants, we reasoned that Southern analysis might be the method of choice: DNA and RNA can be isolated from the same cell culture; DNA can then be digested with *PvuII* and *BamHI*, which cleave 5’ and 3’ of (*A*) and the endogenous *RBCS2* gene so that complete transgenes all have a defined size independent of their site of integration in the genome; and nonintact transgenes generate bands outside of this defined region. Quantification of this defined band relative to a band derived from the endogenous *RBCS2* gene on the same lane of digested DNA gives us the number of intacty integrated transgenes per endogenous gene. To test the feasibility of this approach, defined numbers of colonies derived from strains containing or lacking a single-copy *ble* transgene were scraped from agar plates and inoculated at different ratios in liquid culture. DNA was isolated (as described in reference 12), and the *ble* transgene/endogenous
**RBCS2** gene ratio was determined as outlined above. This ratio correlated well with the fraction of ble transgene-containing colonies (Fig. 1B). Thus, signals for both ble mRNA and intact ble transgenes could be quantified in cotransformant pools. Taking the signal for ble mRNA (corrected for loading) relative to the transgene/endogenous gene ratio allows for a quantitative measure of how many transgenes in pools of cotransformants are indeed expressed. By using large numbers of cotransformants, position effects leading to variations in transgene expression levels should be averaged out. To get an estimate of the minimal number of cotransformants that needed to be pooled in order to average out position effects, we used this new assay to analyze relative transgene expression in a total of 240 cotransformants that were generated with the ARG7 gene (0.2 μg plasmid) and an AR-ble construct (1 μg plasmid). Average ble expression varied tremendously among 12 pools of 20 cotransformants each. Strong variation was still observed in pools of 40, 80, and 120 cotransformants. Only when all 240 cotransformants were pooled did the experimentally determined average transgene expression reach the average calculated from the 12 pools of 20 cotransformants; i.e., variation was averaged out (see Fig. S1 in the supplemental material).

**cis**-regulatory elements of *Chlamydomonas* promoters have been investigated earlier by analyzing pools of cotransformants (e.g., refs 2, 11). However, in those studies variations in cotransformation efficiencies were not taken into account. Because cotransformation efficiencies may vary considerably (20 to 60%) but are quantified in our assay by determining the transgene/endogenous gene ratio, the latter

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**FIG. 1.** Quantification of ble mRNA expression and ble transgene levels in pools of *Chlamydomonas* cotransformants. (A) Analysis of the detection limit for ble transcripts in cotransformant pools. Strains containing an expressing copy of Δ-285AR-ble (Fig. 2) or a nonexpressing copy of R-ble were grown to a density of 5 × 10^6 cells/ml. Cells containing the nonexpressed construct were mixed with the indicated percentage of cells containing the expressed construct. Total cellular RNA was isolated, separated on agarose gels, and transferred to nylon membranes. Membranes were hybridized with a probe detecting the 3' part of the ble coding region, stripped, and rehybridized with a probe generated from the ~1-kb CBLP cDNA (16). Hybridization signals were quantified with the QuantityOne 4.5.1 program (Bio-Rad, Munich, Germany). ble signal values were corrected for unequal loading by values obtained for the constitutively expressed CBLP and plotted against the percentage of ble transgene-expressing cotransformants in the cell culture. Values represent the averages from two independent experiments. (B) Analysis of the correlation between ble transgene signals and number of ble transgene-containing colonies. *Chlamydomonas* cells containing or lacking a single-copy ble transgene were spread onto TAP agar plates, and colonies were grown and counted. About 500 colonies of cells containing the ble transgene were scraped from the agar plates and inoculated alone (100%) or together with the indicated percentage of colonies lacking the ble transgene (20 to 50%). In addition, 800 colonies of the strain lacking the ble transgene were inoculated (0%). After overnight growth in liquid TAP medium, total cellular DNA was extracted, digested with PvuII and BamHI, separated on agarose gels, transferred to nylon membranes, and hybridized with probes against ble or RBCS2, ble and RBCS2 signals were quantified, and ble signal values, corrected for loading by the RBCS2 signal, were plotted against the percentage of ble transgene-containing colonies.
FIG. 2. Analysis of heat shock inducibility and activation of transgene expression by AR-ble constructs. The ble gene is shown as a black bar. Gray lines indicate RBCS2 sequences, i.e., the promoter including 182 bp upstream from the transcriptional start site (+1), the 5’ untranslated region, the first intron, and the 3’ untranslated region. HSP70A promoter sequences were cloned upstream from the R promoter in the orientation (→) that resulted in most efficient activation of the R promoter (14). A promoter deletion end points are given relative to the translational start codon. The transcriptional start site indicated (+1) is that of promoter part PA1 situated 89 bp upstream from the translational start codon (15). Sequence motifs highlighted within the A promoter are a CCAAT box (C), three inverted CCAAT boxes (Ci), four HSEs (I to IV, large black boxes (8), and the TATA box (T, small black box). Gray boxes designate mutated HSEs/TATA, which destroy the canonical nGAAnt/TTCn and TATAA motifs as follows: HSE1, GCGTCCAGAA GGCGCCATACGG→GagaggGgGgGCATACGG; HSE2, GGGGAAGCTGGAGGgGCGtATGG→GGGGAATAgatGgGgCtGCA TGG; HSE3, AGGAAGCTACGAGACTG→AtgGGAAGGACTG; HSE4, GGGGAAGCGCGGACGGTTCGAGAAAGACTGAGGGG→GCCat GGGCGGCCGAGATATAG; and TATA, GGGTATAAAG→GGGAgcAg (underlining indicates the position of the motif within a sequence, boldfaced nucleotides match the canonical motif, wild-type sequences are in uppercase, and introduced mutations are in lowercase). Constructs are drawn to scale; to accommodate −843, it is drawn as a hairpin. For the analysis of heat shock inducibility, 500 zeocin-resistant transformants for each experiment were grown overnight in continuous light; half of the cells were harvested directly (CL), the other half were heat shocked (HS) at 40°C for 30 min. Total RNA was extracted, separated on agarose gels, and transferred to nylon membranes. Membranes were hybridized with the ble probe, stripped, and rehybridized with the CBLP probe. ble signals, including both R and A promoter-derived messages, were quantified and corrected for loading by the CBLP signal. Total ble signal values from heat-shocked cells were divided by the values from nonstressed cells. Given are the averages from three independent experiments ± standard errors of the means. For expression analysis, at least 260 cotransformants were grown overnight in liquid TAP medium. Total RNA and DNA were extracted, separated on agarose gels, and transferred to nylon membranes. Prior to electrophoresis, DNA was digested with PvuII and BamHI. RNA blots were hybridized with the ble probe, stripped, and rehybridized with the CBLP probe. Quantified ble signals were corrected for loading by the CBLP signal. DNA gel blots were hybridized with the ble probe, stripped, and rehybridized with a 263-bp probe derived from sequences upstream from the RBCS2 regulatory sequences (8). Quantified ble signals were corrected for loading by the RBCS2 signal. Corrected ble signal values from RNA blots were divided by corrected ble signal values from DNA blots. Ratios obtained for each construct type were then divided by that from the R-ble construct (pSP115 [9]), which was arbitrarily set to 1.0. Given are the averages from three to eight experiments ± standard errors of the means.
represents a notable improvement for promoter analyses in *Chlamydomonas*.

When we used this new assay to determine the efficiency of A promoter derivatives in activating transgene expression, we found that differences between the constructs were much more pronounced than those obtained by counting zeocin-resistant colonies generated by direct transformation with R-ble/AR-ble constructs (14). There, the ratio for constructs Δ-843AR-ble:Δ-285AR-ble:R-ble was 3.6:2.6:1, whereas it was 26.7:5.6:1 with the new assay (Fig. 2). Using the R promoter-driven aadA gene, conferring resistance to spectinomycin, Cerutti et al. found that about half of the transformants initially selected on the drug became transcriptionally silenced when grown under nonselective conditions for a few weeks (3). Thus, it is possible that in the direct transformation experiment the active state of R-ble transgenes is favored by the selective pressure, which is not the case in cotransformation experiments. If the A promoter generally maintained a transgene in the active state, this would explain why AR-ble compared to R-ble constructs gave higher values in cotransformation than in direct transformation assays.

As judged from the 26.7-fold stimulation of construct Δ-843AR-ble compared to the 5.6-fold stimulation of Δ-285AR-ble relative to R-ble, the distal region appears to contribute more than three-quarters of the maximal stimulatory effect of the A promoter (Fig. 2). Mutation of HSE4 in the distal region (construct Δ-843-hse4) reduced the activation effect of the A promoter to 12.2-fold, suggesting a central role for HSE4 in mediating the effect. However, as the distal region mutated in HSE4 still was as efficient in transgene activation as the proximal region (both contributing about sixfold), additional sequence motifs within the distal region appear to be involved, e.g., the CCAAT boxes. Deletion of HSE3 in constructs Δ-222 and Δ-197 and mutation of HSE3 in Δ-285-hse3 resulted in at most a weak decrease in the activation effect compared with the parental construct Δ-285. Deletion and mutation of HSE2 (constructs Δ-162 and Δ-285-hse2) dramatically reduced the activation effect mediated by the proximal A promoter region, suggesting a central role for HSE2 in transgene activation. Activation rates remained low when HSE1 also was removed in construct Δ-143. Mutation of HSE1 and the TATA box in constructs Δ-285-hse1 and Δ-285-tata, respectively, resulted in a reduction of the activation effect by a factor of about 2 compared to the parental construct Δ-285, suggesting a contribution of both sequence motifs to the activation effect. Combining mutations in HSE1 and HSE2 in construct Δ-285-hse1/2 completely abolished the activation effect of the proximal region. Taken together, our data suggest that HSE4 and HSE2 play central roles in transgene activation; HSE1 and HSE3 appear to enhance the effect of HSE2 but cannot substitute for its loss. In addition, the activation effect mediated by the promoter-proximal HSEs appears to require a functional TATA box.

These findings suggest a central role in transgene activation of heat shock factors, the transcription factors that bind to HSEs and mediate the heat shock response (1). If this holds true, A promoter derivatives with low transgene-activating activities would also be expected to be impaired in conferring heat shock inducibility to the transgene. To test this, constructs were directly transformed into *Chlamydomonas* and pools of at least 500 independent zeocin-resistant transformants were tested for heat shock inducibility of the ble gene. As shown in Fig. 2, all A promoter derivatives containing intact HSE1, HSE2, and the TATA box, which all activated ble transgene expression at least 4.5-fold, also conferred heat shock inducibility by at least 2.9-fold. In contrast, when the A promoter, in addition to the TATA box, contained only HSE1 or no HSEs or when HSE1, HSE2, or HSE1 and HSE2 were mutated, the drop in transgene activation to values of between 0.8- and 3.0-fold was accompanied by a drop of heat shock inducibility to values of between 0.8- and 1.8-fold. Mutating the TATA box, which reduced transgene activation about twofold, also reduced heat shock inducibility by a factor of about 2 (Fig. 2). We suppose that in the absence of the regular TATA box, transgene activation as well as heat shock-induced transcriptional activation was conferred with reduced efficiency by a TATA-like element of the downstream P45 promoter (15). Taking the data together, for the region proximal to HSE3, we do find a correlation between the abilities of an A promoter derivative to activate transgene expression and to mediate heat shock inducibility.

In summary, our data suggest that the ability of the A promoter to activate transgene expression is mediated largely by the concerted action of heat shock factors and TATA-binding factors. As proposed previously (14), these factors might constitutively occupy the promoter and by recruiting chromatin-remodeling activities keep surrounding chromatin in an open state. The mapping of two strong, constitutive nuclease-hypersensitive sites to HSE4 and to the region between the TATA box and HSE1 to HSE3 supports this idea (8).

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