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The *HSP70A* promoter as a tool for the improved expression of transgenes in *Chlamydomonas*

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Summary

The *Chlamydomonas reinhardtii HSP70A* promoter can be induced by both heat shock and light. Several characteristics of this promoter suggest its usefulness as a tool for improved transgene expression in this alga. (i) It may by itself confer high inducibility to a transgene. Fusion of the *HSP70A* promoter to reporter genes *HSP70B* or *ARS* yields high levels of transgene product that, as shown for ARS, may accumulate when repeated cycles of heat shock induction are applied. (ii) It activates other promoters. Using *HSP70B* as a reporter gene, we show that the *HSP70A* promoter serves as a transcriptional activator when placed upstream of the promoters *RBCS2*, *β_2TUB* and *HSP70B*. Activation of these promoters was observed both under basal conditions and upon light induction. In addition, transformation rates obtained for the eubacterial resistance gene *aadA* were significantly increased, when expression of this gene was controlled by the *HSP70A–RBCS2* promoter fusion as compared to the *RBCS2* promoter alone.

Introduction

Chlamydomonas has long been used as a model system for studying photosynthesis, organelle biogenesis and the biosynthesis and function of flagella, mainly due to its well-defined genetics (Harris, 1989). The development of DNA transformation techniques for this organism has contributed significantly to the growing interest in *Chlamydomonas* as a model organism: *Chlamydomonas reinhardtii* is the first photosynthetic eukaryote that allows stable transformation of the nuclear (Debuchy *et al.*, 1989; Kindle *et al.*, 1989), chloroplast (Boynton *et al.*, 1988) and mitochondrial (Randolph-Anderson *et al.*, 1993) genomes. Once introduced into the cell, transforming DNA is readily delivered to the nucleus, where it integrates mostly non-homologously at random sites into the genome (Kindle and Sodeinde, 1994). The selectable markers used so far for most studies represent *Chlamydomonas* genes that complement mutant phenotypes to wild type upon transformation. Examples of such genes are *ARG7* (Debuchy *et al.*, 1989), *NIT1* (Kindle *et al.*, 1989), *OEE1* (Mayfield and Kindle, 1990), *ATPC* (Smart and Selman, 1993), *NIC-7* (Ferris, 1995) and *THI-10* (Ferris, 1995). Transformation with any such gene requires crossing of the respective mutation into the recipient strain of interest. The *CRY1* gene of *C. reinhardtii*, coding for a mutated version of the ribosomal S14 protein, conferring resistance to the translation inhibitors emetine and cryptopleurine, was the first dominant selectable marker allowing direct transformation of any *Chlamydomonas* strain (Nelson *et al.*, 1994). Only recently, the bacterial *ble* gene of *Streptoalloteichus hindustanus*, conferring resistance to phleomycin and its derivatives, was shown to be expressed in *Chlamydomonas* and to segregate with the resistance phenotype (Stevens *et al.*, 1996). Expression of the *ble* gene was driven by the *RBCS2* promoter of *C. reinhardtii*. The low transformation efficiencies obtained upon phleomycin selection were significantly increased by the introduction of the first *RBCS2* intron into the *ble* gene (Lumbreras *et al.*, 1998). The bacterial *aadA* gene, fused to the *RBCS2* promoter, yielded spectinomycin resistant transformants only at a very low frequency (Cerutti *et al.*, 1997b). Although the resistance phenotype was demonstrated to segregate with the transgene, half of the transformants exhibited an unstable phenotype (Cerutti *et al.*, 1997a). The low transformation efficiencies and the unstable phenotype were attributed to transcript instability and an epigenetic silencing mechanism, respectively.

In general, the difficulties encountered with the expression of foreign genes in *C. reinhardtii* and especially the

lack of strong promoter systems have been a major drawback to the further development of *Chlamydomonas* as a model organism. The problems with foreign gene expression were circumvented by using the above-mentioned *Chlamydomonas* genes as selectable markers and the *Chlamydomonas ARS* gene encoding arylsulfatase as a reporter gene (Davies *et al.*, 1992; Ohresser *et al.*, 1997). However, the need for a strong promoter system still persisted. All attempts to express (foreign) genes under the control of the CaMV 35S promoter in *C. reinhardtii* have failed thus far (Blankenship and Kindle, 1992; Day *et al.*, 1990). *Chlamydomonas* promoters used for the expression of both homologous and heterologous genes have produced rather diverse results: in the vast majority of cases studied, only very low levels of transgene expression (below 10%) were observed both for *Chlamydomonas* wild-type genes and for chimeric genes composed of promoters and coding regions derived from different sources (Blankenship and Kindle, 1992; Davies and Grossman, 1994; Davies *et al.*, 1992; Kindle *et al.*, 1989; Kozminski *et al.*, 1993). However, transgene expression levels similar to those observed for the endogenous gene have also been observed (Diener *et al.*, 1990; Hippler *et al.*, 1998; Smart and Selman, 1993). For example, epitope-tagged α-tubulin, encoded by a gene transcribed by the *RBCS2* promoter, accounted for 40–70% of the total cellular α-tubulin, suggesting a high transcriptional activity of the constitutive *RBCS2* promoter (Kozminski *et al.*, 1993). This result initiated the fusion of the *RBCS2* promoter to a number of genes, among them *ble* (Stevens *et al.*, 1996), *aadA* (Cerutti *et al.*, 1997b), the *Chlamydomonas* photolyase homologue *CPH1* (G.D. Small, personal communication) and *HSP70B* (this study). Although expression was observed in some cases, the expression levels obtained were far below those expected.

In this study we have created synthetic promoters by fusing the *HSP70A* promoter to other *Chlamydomonas* promoters and present an analysis of the expression of several transgenes controlled by these synthetic promoters. We show that the *HSP70A* promoter serves as a transcriptional activator of promoters *RBCS2*, β_2*TUB* and *HSP70B* in the *HSP70B* gene context, leading to a clearly detectable basal expression under non-inducing conditions and high level expression under inducing conditions. Moreover, we show that the *HSP70A* promoter confers light inducibility to promoters *RBCS2* and β_2*TUB*, which are normally not inducible by light. Furthermore, we demonstrate that the transformation efficiency is significantly increased when the *HSP70A* promoter is fused upstream to the *RBCS2* promoter, driving expression of the *aadA* cassette. Finally, using an *HSP70A* promoter/*ARS* gene fusion, we show that using periodically applied heat shocks, a stable gene product may accumulate to high concentrations.

Results

Promoter constructs and their performance in transformants exhibiting transgene expression

Our original goal was to overexpress the *HSP70B* gene (Drzymalla *et al.*, 1996; von Gromoff *et al.*, 1989) in order to perform a gene function analysis based on a phenotype that may result from *HSP70B* overexpression (Schroda *et al.*, 1999). To distinguish between endogenous gene and transgene, a genomic copy of the *HSP70B* gene had been converted into a reporter gene by the insertion of a 213 bp tag sequence into its 3′ UTR (*HSP70B-tag*), and by the introduction of the coding region for a C-terminal hexahistidine tag (Schroda *et al.*, 1999). Since in all constructs used, *HSP70B-tag* was located on the same vector and next to the selection gene *ARG7* (Debuchy *et al.*, 1989), between 60 and 80% of the Arg^+ transformants contained the tag sequence in their genome (data not shown).

Various promoter constructs in front of *HSP70B-tag* were tested in Arg^+ transformants generated with linearized plasmid DNAs of pCB706, pCB739, pCB720 and pCB740 (Figure 1). From each transformation experiment, 30 randomly picked Arg^+ transformants were analyzed for transgene expression by the RNA dot blot method. Constructs harbouring the *HSP70B* promoter with 1 kb of upstream sequences (pCB706), after heat shock, yielded one transformant expressing *HSP70B* at a low level (Figure 2a). Neither the enlargement of the *HSP70B* upstream sequences (up to 3.6 kb), nor the introduction of further deletions into the *HSP70B* promoter led to an increase in the number of transformants that expressed the transgene at detectable levels (data not shown). Furthermore, the *RBCS2* promoter (*R*) (Goldschmidt-Clermont and Rahire, 1986) in construct pCB739 gave rise to only a very few transformants that exhibited low expression levels. In contrast, the use of the *HSP70A* promoter (*A*) (pCB720) resulted in transformants of which about one-third, after heat shock, exhibited elevated levels of transgene-specific mRNA. A similar yield in expressing transformants after heat shock was observed when an *AR* promoter fusion (pCB740) was employed. Note that in this case at least one transformant yielded very high transgene expression after heat shock (Figure 2a), a result which was subsequently confirmed by additional experiments where the percentage of transformants exhibiting very high expression levels was even higher (data not shown).

To test whether the high levels of *HSP70B-tag* mRNA also gave rise to transgenic HSP70B protein, Western blots of whole cell protein were immunodecorated with antibodies raised against the HSP70B protein, and with antibodies directed against the hexahistidine tag present at the C terminus of the transgenic HSP70B protein (Figure 2b). In two independent transformants generated with

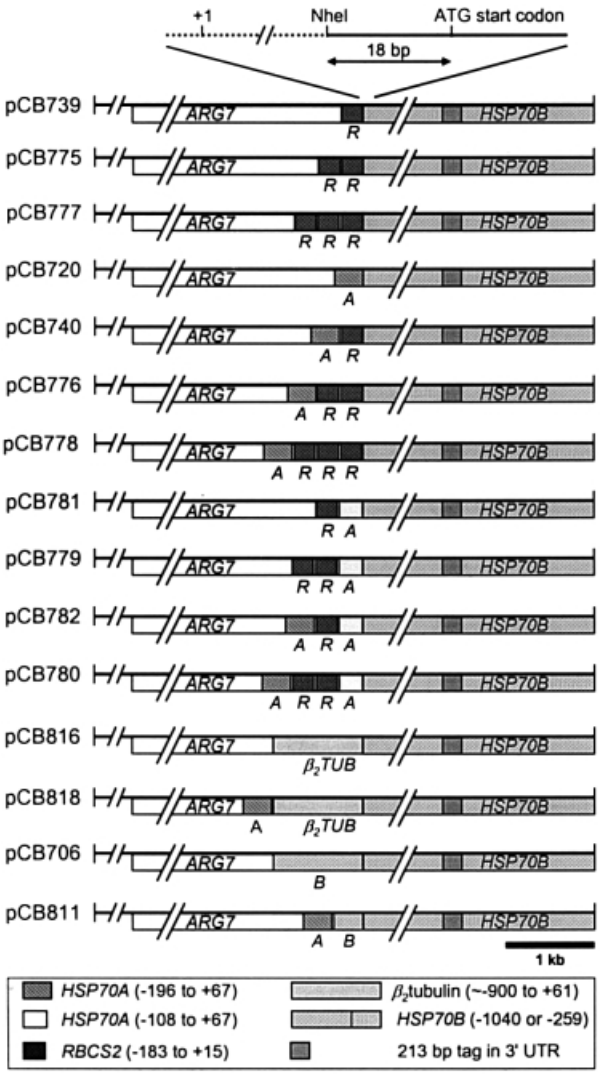


Figure 1. Promoter constructs used in this study that are fused to the *HSP70B* gene.
The *HSP70B* gene (grey box) has been converted into a reporter gene by the insertion of the 213 bp tag (stippled box) into the 3′ UTR. All constructs also harbour the *ARG7* gene (white box) for the selection of arginine prototrophic transformants. The symbols used to denote the various promoters including the 5′ UTR and the tag sequence are shown below the constructs. The promoters of genes *HSP70A*, *HSP70B* and *RBCS2* are designated with *A, B* and *R*, respectively.

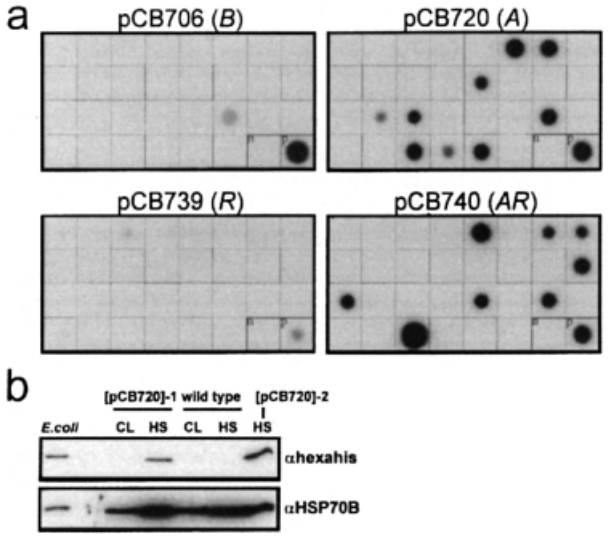


Figure 2. Expression assay of randomly selected clones of strain cw15–302 transformed with the constructs indicated (see Figure 1).
(a) RNA dot blot analysis. Transformants were selected for arginine prototrophy conferred by the *ARG7* gene located on the transforming constructs. The vectors also contained the *HSP70B* reporter gene (*HSP70B-tag*) driven by the Δ-1040 *HSP70B* promoter (pCB706), the Δ-183 *RBCS2* promoter (pCB739), the Δ-196 *HSP70A* promoter (pCB720) or the Δ-196 *HSP70A*/Δ-183 *RBCS2* tandem promoter (pCB740), respectively. Transformants were grown in continuous light and RNA was isolated directly (pCB739 and respective controls – the lower transgene mRNA level of the positive control strain when compared to the other panels reflects the absence of induction by heat shock) or after a 30 min heat shock at 40°C. Strains CF184 (transformed with pCB740) and CF185 (transformed with *ARG7* alone) were used as positive (p) and negative controls (n), respectively. *HSP70B* reporter gene expression was detected by hybridization with the 213 bp tag probe.
(b) Analysis of the expression of transgenic HSP70B protein. Strain CC-124 (wild type) and two transformants with construct pCB720 were grown in continuous light (CL) or heat-shocked for 2.5 h (HS). Whole cell protein isolated from these and from an *E. coli* strain expressing hexahistidine-tagged HSP70B, was separated by SDS-PAGE (10 μg per lane) and transferred to nitrocellulose membranes. Membranes were immunodecorated with antibodies raised against the hexahistidine tag (top gel) or against the HSP70B protein (bottom gel). Since both antisera detect the entire HSP70B protein present in *E. coli*, exposure times giving equal band intensities for the *E. coli* HSP70B allow for a direct assessment of the amount of transgenic HSP70B (top gel) and of total cellular HSP70B (bottom gel).

pCB720, transgenic HSP70B protein was absent under non-inducing conditions, however, this protein accounted for about 15% ([pCB720]-1) and 60% ([pCB720]-2) of total cellular HSP70B protein after 2.5 h of heat shock. When the *AR* promoter was used to drive the *HSP70B-tag* gene, twofold higher HSP70B protein levels were observed (Schroda *et al.*, 1999). These results provided a first indication for the utility of the *HSP70A* promoter, alone or in combination with the *RBCS2* promoter, for the expression of transgenes.

In Figure 3, a Northern blot analysis of selected individual transformants that exhibited expression in RNA dot blot studies is shown. Clones selected for Northern analysis were randomly chosen from a set of transformants that exhibited equally high expression levels (except for those harbouring one or two *R* promoters (pCB739 and pCB775), where only one transformant in each case showed elevated signal intensity). Thus, the transformants analyzed are considered to represent those with maximum transgene expression obtainable for the respective constructs. Each selected transformant was subjected to Southern gel blot analysis to confirm integration of the entire chimeric transgene. Most likely due to the low amount of DNA used for transformation

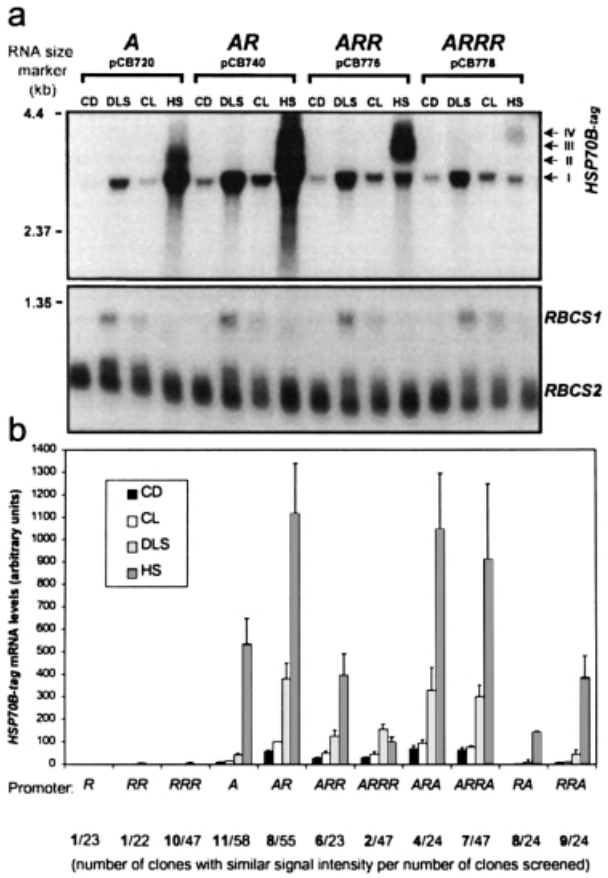


Figure 3. Expression of the *HSP70B* reporter gene driven by selected combinations of the Δ-196/Δ-108 *HSP70A* (*A*) and Δ-183 *RBCS2* (*R*) promoters in single clone transformants.

(a) Northern blot hybridization of total RNA (10 μg per lane) isolated from cells which were either kept for 16 h in the dark (CD), exposed to a 1 h incubation at low light intensities (40 μE $m^{-1} s^{-1}$) after the dark period (DLS), maintained in continuous light (CL), or heat-shocked for 40 min at 40°C in continuous light (HS). The *HSP70B* reporter gene transcripts were detected with the 213 bp tag probe. (I) designates transcripts originating from the transcriptional start site of the promoter closest to the *HSP70B* gene (~ 2950 bp); (II, III and IV) in this order designate transcripts originating from the transcriptional start site of the *HSP70A* promoter with increasing numbers of interspersed *RBCS2* promoters (~ 3200 bp, ~ 3450 bp and ~ 3700 bp). For a loading control, blots were stripped and rehybridized with a probe of the *RBCS* coding region that detects the transcripts of both *RBCS* genes. The left ordinate gives the size of the RNA marker.

(b) Quantitative evaluation of the *HSP70B* reporter gene mRNA levels from Northern blots hybridized with the tag probe. Signal intensities were corrected for unequal loading using the respective *RBCS2* signal. The data were obtained from two to seven independent experiments performed with one to three individual transformants for each construct. Values given are relative to the CL value of a pCB740 transformant (*AR*), which was arbitrarily set to 100. Expression levels are shown as the mean and standard error of the mean (SEM). The number of clones that exhibited an equal signal intensity in RNA dot blot experiments as the ones chosen for gel blot analyses is indicated over the total number of transformants screened.

(100 ng per 10^8 cells), each transformant analyzed contained only one or, at the most, two intact copies of the transgene (data not shown).

Analysis of a transformant with the *HSP70A* promoter (pCB720) showed a very low level of expression when incubated in darkness and a slightly increased level in the light. Expression was inducible by light and heat shock, which confirms previous results (Kropat *et al.*, 1995; Kropat *et al.*, 1997; von Gromoff *et al.*, 1989). The *AR* promoter fusion (pCB740) exhibited several traits of interest. (i) The transformants showed substantial basal level expression in darkness and in light. (ii) This promoter construct also showed a distinct light inducibility, reaching levels of about one-third of those seen after heat shock treatment (Figure 3a,b). The size of the transcripts under basal conditions and after light induction were identical to those of construct pCB720, suggesting that they started within the promoter proximal to the *HSP70B* gene, i.e. the *R* promoter. Note that the *R* promoter itself is at most very weakly inducible by light (Figures 3a and 4, lower panels, and Goldschmidt-Clermont and Rahire, 1986; Goldschmidt-Clermont, 1986). Quantification of *RBCS2* transcript levels in 20 independent transformants with a cw15–302 strain background have revealed, on average, a 23% increase upon shift of cultures to light and a 13% decrease after heat shock. (iii) The fused *AR* promoter was strongly inducible by heat shock. The increased size of the transcripts seen under heat shock conditions suggests that these transcripts initiated from the *A* promoter. Weaker exposures revealed that, during heat shock, expression from the *R* promoter decreased only slightly.

These results were confirmed and extended by constructs that harboured 2 (pCB776) and 3 (pCB778) *R* promoters between the *A* promoter and the *HSP70B-tag* reporter gene. As seen before for the *AR* construct, the *A* promoter conferred elevated basal level expression and light inducibility to the *R* promoter closest to the *HSP70B-tag* gene. The size of the heat shock-induced mRNA increased with the insertion of multiple *R* promoters, confirming our assumption that transcription under these conditions initiates from the *A* promoter. We also noted that expression from the *A* promoter became weaker with an increasing distance of this promoter from the reporter gene (Figure 3a). Surprisingly, no light inducible transcripts originating from the *A* promoter were detected in *AR* constructs. When *R* promoter sequences were used as a probe for hybridization, transcripts were detected only in heat-shocked transformants that harboured constructs *AR*, *ARR* and *ARRR* (data not shown). This result confirmed that, in these constructs, the transcriptional start under basal conditions and light induction was at the proximal *R* promoter, whereas upon heat shock it was at the distal *A* promoter. From these results we conclude that the *A* promoter confers an elevated basal activity as well as inducibility by light on the otherwise only very weakly active *R* promoter. The fused promoters thus exhibit new, synthetic properties.

The results from an analysis of additional constructs are summarized in Figure 3(b). Placing one or two copies of the *R* promoter in front of the *A* promoter yielded constructs that in their regulatory properties did not significantly differ from those with *A* alone. However, constructs in which one or two *R* promoters were sandwiched between two *A* promoters exhibited expression patterns similar to those of the *AR* setup. Here, the transcription start sites used for basal and light-induced expression were within the *A* promoter closest to the *HSP70B-tag* gene (data not shown). Upon heat shock, both *A* promoters were transcriptionally active, however, the one proximal to the *HSP70B-tag* gene dominated over the distal *A* promoter (data not shown). The analysis of transformants that harbour two or three *R* promoters in front of the reporter gene confirmed the very low level of expression obtained with a single *R* promoter (Figures 2a and 3b).

In summary, analysis of the constructs revealed that only the setup in which an *A* promoter is located upstream of an *R* promoter may confer new regulatory properties; the reverse order, i.e. *RA*, exhibited only the properties of the *A* promoter. The *AR* promoter fusion thus appears to be a promoter system suited for cases where an elevated expression of transgenes as well as transient inducibility are desired.

The activating effect of the HSP70A promoter is not restricted to the RBCS2 promoter

Since transformants that harbour the *HSP70B-tag* reporter gene under control of its own promoter with one exception (one out of 700 co-transformants) yielded only clones that expressed the *HSP70B-tag* gene weakly (Figure 2a), we could test whether the *A* promoter had a stimulatory effect on the *HSP70B* promoter (*B*). Cloning of the *A* promoter in front of the *B* promoter (*AB*) resulted in the clearly detectable expression of the transgene (Figure 4). As in the case of the *AR* fusion promoters, elevated basal as well as light-induced expression originated from the promoter proximal to the reporter gene (*B*). Note that the *B* promoter is light and heat shock-inducible in its endogenous context (von Gromoff *et al.*, 1989), but exhibits this property at detectable levels in a transgene context only when fused with the *A* promoter. In the case of the *AB* promoter fusion, a low level of mRNA originating from the *A* promoter could also be detected after light induction. Heat shock in this fusion construct resulted in the activation of both the *B* and the *A* promoters.

Furthermore, the β_2*TUB* promoter (Davies *et al.*, 1992), showing only weak activity in the great majority of transformants (a representative transformant is shown in Figure 4), is subject to activation by the *A* promoter (*A*β_2*TUB*). The expression pattern observed, i.e. elevated basal levels and light inducibility, resembles that of the *AR* and *AB* promoter fusions. The slight light inducibility of the β_2*TUB* promoter in the *HSP70B-tag* gene context (about 1.8-fold) was contrasted by a 5.4-fold increase in mRNA upon light induction of the *A*β_2*TUB* promoter fusion, which is about the same as that observed with the *A* promoter alone. Again, the transcripts observed appear to originate from the β_2*TUB* promoter. Heat shock of transformants with the *A*β_2*TUB* fusion promoter did not result in elevated transcript levels. This is in contrast to the *AR* and *AB* fusion promoters. However, in the former case, transcripts starting from the *A* promoter harbour about 900 bp of extra sequences of the β_2*TUB* promoter at their 5′ ends, and we speculate that this may either result in instability of the resulting mRNA, or in termination of transcription due to polyadenylation signal-like motifs contained within the 900 bp of β_2*TUB* promoter sequences.

In summary, these results show that the *HSP70A* promoter may confer new properties on various *Chlamydomonas* promoters, activating them to elevated basal expression as well as providing them with inducibility by light.

Use of the AR *promoter fusion for the expression of the eubacterial* aadA *gene in* Chlamydomonas

To test the effect of the *AR* promoter fusion on the expression of foreign genes in *C. reinhardtii*, we generated a construct that harbours this promoter in front of the *aadA* gene, conferring resistance to spectinomycin (Cerutti *et al.*, 1997b).

When an *AR-aadA* construct (pCB801) was used, the relative number of spectinomycin resistant colonies was more than 2.5-fold higher than with an *R-aadA* construct (pCB800). This result was consistent for the three different strains used (Table 1). In Figure 5, pools of spectinomycin resistant transformants that were obtained by direct selection (90 $\mu g\,ml^{-1}$ spectinomycin) and kept under selective conditions (45 $\mu g\,ml^{-1}$ spectinomycin) were analyzed for *aadA* mRNA levels. In continuous light, equally low transcript levels present as a diffuse signal were detected for both the *R-aadA* construct (pCB800) (see also Cerutti *et al.*, 1997b) and the *AR-aadA* construct (pCB801), therefore not accounting for the observed differences in transformation rates (Table 1). In contrast, *aadA* mRNA encoded by *AR-aadA* was clearly detectable as distinct bands under conditions of light or heat shock induction. As judged by their size of approximately 1.4 kb and by hybridization with *R* promoter sequences, transcripts under these conditions initiated from the *HSP70A* promoter (Figure 5 and data not shown). Therefore, it is not clear whether the difference in the relative number of transformants obtained under continuous light with both constructs (Table 1) can be attributed to an increased

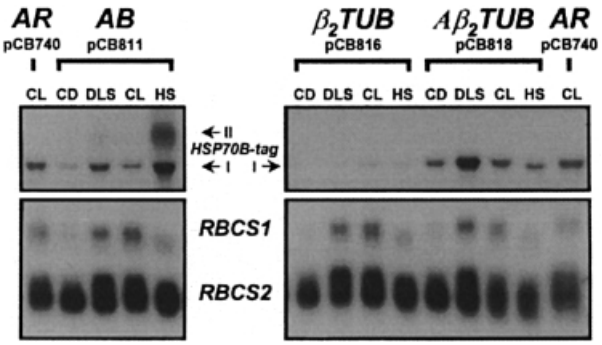


Figure 4. Expression of the *HSP70B* reporter gene driven by the promoter of the β_2*TUB* gene or by fusions of the *HSP70A* promoter (*A*) with promoters *RBCS2* (*R*), *HSP70B* (*B*) and β_2*TUB.HSP70B* reporter gene mRNA levels were detected with the 213 bp tag probe.
Constructs harbouring only the *HSP70B* promoter (Figure 1) gave rise to only barely detectable RNA after heat shock treatment (Figure 2a). (I) designates transcripts originating from the transcriptional start sites of the *RBCS2, HSP70B* and β_2*TUB* promoters, and (II) those originating from the *HSP70A* transcriptional start site. Blots were stripped and rehybridized with a probe for the *RBCS* coding region as a loading control. Culture conditions and symbols used were as described in Figure 3.

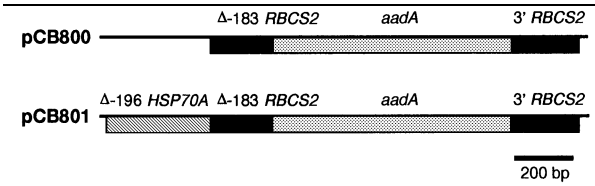
transcription rate of the *AR-aadA* construct or to other properties of the *AR* promoter.

Transgene expression in response to single and repeated activation of the HSP70A *promoter*

To test the utility of the *HSP70A* promoter for the regulated expression of a transgene, we employed fusions of the *A* promoter with a promoter-less *ARS* gene, encoding arylsulfatase. This *Chlamydomonas* gene, encoding a periplasmic protein, has been used previously as a reporter gene in *C. reinhardtii* (Davies and Grossman, 1994; Davies *et al.*, 1992; Ohresser *et al.*, 1997; Quinn and Merchant, 1995). The endogenous gene is completely silent in the presence of sulfate in the medium (de Hostos *et al.*, 1989). In transformants with an *A–ARS* fusion construct, the *ARS* gene was induced after a shift of cells from 23°C to 40°C for 1 h (Figure 6). First, a rapid and transient accumulation of *ARS* mRNA was observed which peaked around 60 min after the temperature shift (Figure 6a). The subsequent disappearance of *ARS* mRNA occurred with a half-life time of approximately 15 min and is accounted for by a switching-off of the *HSP70A* promoter after 60 min and the intrinsic instability of *ARS* mRNA (de Hostos *et al.*, 1989; Quinn and Merchant, 1995). ARS enzyme activity started to increase from very low pre-induction levels with a delay of about 30 min. Within 90 min after induction, an about 26-fold rise in enzyme activity was observed. The subsequent gradual decrease in the specific activity of this very stable enzyme (de Hostos *et al.*, 1988) may be accounted for by an increase in cell mass. These results

Table 1. Transformation of *Chlamydomonas* with the bacterial *aadA* gene

Transformed strain	Spc^r colonies[a] (pCB801 relative to pCB800)
CC-124	2.8 ± 0.3 (n = 7)
CF97	3.2 ± 0.7 (n = 2)
CF98	2.5 (n = 1)



[a]The average number of transformants per 10^8 cells obtained out of 20 plates, i.e., 14 transformation experiments was 21 ± 4 for pCB800 and 60 ± 16 for pCB801 (control without DNA: 5 ± 2).

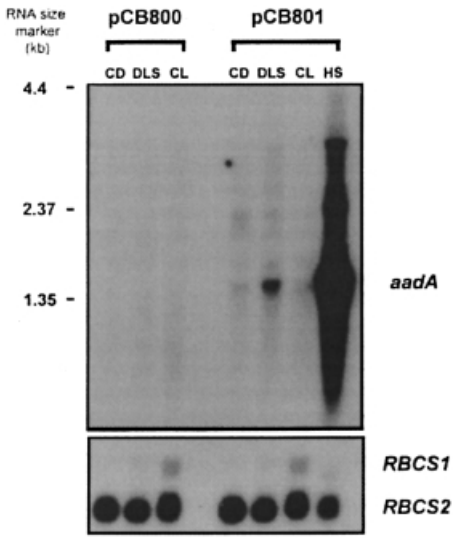


Figure 5. Expression analysis of spectinomycin resistant cells generated by transformation with the *aadA* gene transcribed by the *RBCS2* promoter alone (pCB800) or the *HSP70A-RBCS2* promoter fusion (pCB801).
For Northern blot analysis, total RNA (10 μg per lane) isolated from a pool of 35 Spc^r transformants per construct was hybridized with the *aadA* gene probe. Blots were stripped and rehybridized with the *RBCS* coding region as a loading control. Autoradiographs were exposed for 2 days (*aadA* hybridization) and overnight (*RBCS* hybridization), respectively. Basal and inducing conditions were as described in Figure 3. The left ordinate gives the size of the RNA marker.

confirm that the transcriptional activity of the *HSP70A* promoter is very low under non-inducing conditions, consistent with results obtained with the *HSP70B* transgene (Figure 3). The rapid and high level expression of the *ARS* reporter gene after application of heat stress illustrates the utility of this promoter for the transient synthesis of transgene products.

Next, we optimized the conditions for a high yield of product from a transgene driven by the *HSP70A* promoter.

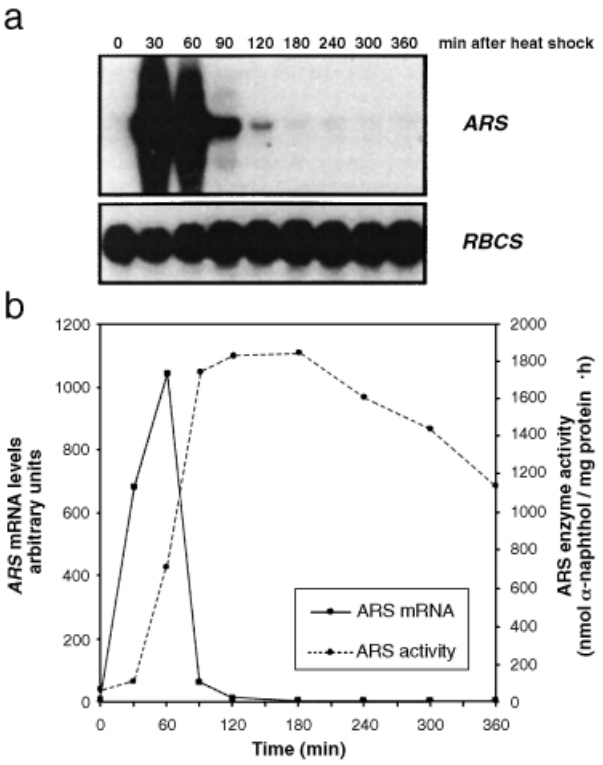


Figure 6. Induction kinetics of an *HSP70A* promoter-*ARS* gene fusion at the level of *ARS* mRNA accumulation and ARS enzyme activity.
(a) Northern blot analysis of total RNA (10 μg per lane) isolated from a transformant generated by co-transformation of pCB803 (*A-ARS*) with pCB412 (containing the selection gene *ARG7*), using the *ARS* cDNA as a probe. Construct pCB803 contained the *HSP70A* promoter (–108 to +67) in front of the 7 kb genomic *ARS* gene. Cells were heat-shocked at 40°C for 1 h. Samples were taken at the times indicated and split for isolation of total RNA or determination of ARS enzyme activity.
(b) The *ARS* hybridization signals were quantified and unequal loading was corrected by the *RBCS* signal. ARS enzyme activity was determined by measuring the concentration of α-naphtol made from α-naphtyl sulfate by arylsulfatase at 37°C for 1 h and standardized to total cellular protein.

For this purpose, we first determined the duration of heat stress required for maximum expression of an *A–ARS* fusion. As shown in Figure 7(a), application of a heat-shock for 30 min allowed maximal expression of the *ARS* gene under these conditions. To obtain a high yield in the transgene product, we considered the possibility of repeated heat shock applications. After heat shock and the synthesis of elevated levels of heat shock proteins by stressed cells, the *HSP* genes are turned off by a regulatory system resembling autoregulation (Morimoto, 1993; Shi *et al.*, 1998). The genes are then refractory to another induction for some time. We therefore determined the time required for recovery of induction competence after heat shock. Following a first heat shock of 30 min, a second treatment was applied 2, 3, 4 or 5 h after the end of the first one (Figure 7b). A gradual regaining of induction competence was observed and a complete recovery was seen after 5 h. A second heat shock after 5 h resulted in an *ARS* activity exactly twice that observed after a single heat shock. We conclude that, provided the gene product is stable, the repeated heat-shock activation of a transgene fused to the *HSP70A* promoter in a 5 h rhythm is a valid procedure for the elevated production of desired gene products in *C. reinhardtii*.

Discussion

In this study we demonstrated the capability of the *HSP70A* promoter (*A*) to improve transgene expression in *Chlamydomonas*. In contrast to promoters of genes *HSP70B (B*), *RBCS2* (*R*) and β_2*TUB*, the *A* promoter by itself may, after induction, confer high level expression to transgenes *HSP70B* and *ARS* (Figures 2, 3 and 6). Thus, high levels of stable transgene products may be obtained by periodically applied heat shocks (Figure 7b). Moreover, the *A* promoter, driving expression of the *HSP70B-tag* reporter gene, significantly increased the transcriptional activity of the *B*, *R* and β_2*TUB* promoters when fused upstream of these. In addition, these promoter fusions exhibited new regulatory properties: the normally not light responsive promoters *R* and β_2*TUB* showed a distinct light inducibility when placed behind the *A* promoter.

In the case of the *AR* construct, the expression levels observed under inducing conditions, i.e. light or heat shock, clearly indicate a synergistic effect of the promoters upon each other (Figure 3b). Thus, the expression levels from the *AR* promoter fusion after dark-to-light shift or heat shock exceeded the values expected for an additive effect by a factor of approximately two (Figure 3b). From additional combinations of the *HSP70A* and *RBCS2* promoters (*RR*, *RRR*, *RA*, *RRA*, *ARR*, *ARRR*, *ARA* and *ARRA*) fused to the *HSP70B-tag* gene, the *AR* motif was found to represent the common trait of all synthetic promoters that exhibited significantly increased expression levels under all conditions tested, other than heat shock. As was evident from promoter combinations *RR*, *RRR*, *RA* and *RRA*, the mere fusion of promoters did not lead to increased expression levels or synergistic behaviour *per se*.

Although not mapped precisely, each synthetic promoter under non-inducing conditions and upon dark-to-light shift quantitatively initiated transcription at the promoter proximal to the *HSP70B-tag* gene, independent of this promoter being *A*, *R*, β_2*TUB* or *B*. In contrast, with the exception of the *AB* promoter fusion (the *B* promoter by itself is heat shock-inducible), heat shock-induced transcription only started at the *A* promoter. We conclude that, at least in the *HSP70B-tag* gene context studied here, heat shock-inducibility cannot be conferred from the *A* promoter to the *R* or β_2*TUB* promoters. In contrast, inducibility by dark-to-light shift may be conferred by the *A* promoter

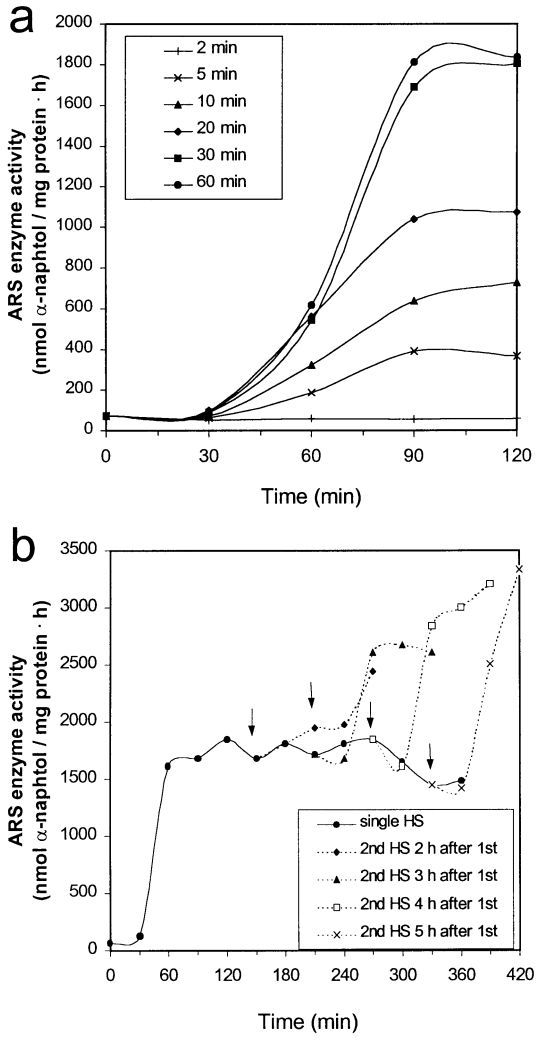


Figure 7. Optimization of *ARS* expression from the *HSP70A* promoter.
(a) Determination of the heat-shock duration required to obtain the full heat shock response. A late-log phase culture of a transformant with the *HSP70A-ARS* fusion construct (pCB803) was distributed equally into six Erlenmeyer flasks (40 ml each) and incubated for 2, 5, 10, 20, 30 and 60 min at 40°C. After this incubation, cultures were shifted back to 23°C. ARS enzyme activity was determined as described in Figure 6.
(b) Determination of the minimum time required between two successive heat shocks for the re-establishment of competence for a full heat shock response. A late-log phase culture of a transformant with the *HSP70A-ARS* fusion construct (pCB803) was distributed equally into five Erlenmeyer flasks (40 ml each), all of which were incubated for 30 min at 40°C and then shifted back to 23°C. Aliquots were exposed to a second heat shock by incubation for 30 min at 40°C 2, 3, 4 and 5 h after the end of the first heat shock (time points indicated by arrows). Samples were taken every 30 min from all aliquots. ARS enzyme activity was determined as described in Figure 6.

onto other promoters over distances as long as 900 bp. This observation indicates that transcriptional activation of the *A* promoter by dark-to-light shift is mediated by mechanisms different from those employed for heat shock, thus confirming previous results (Kropat *et al.*, 1995). A comparable transfer of properties from one promoter to another has been reported in *Chlamydomonas* previously (Quinn and Merchant, 1995). In that particular study, copper-responsive CuRE upstream sequence elements of the *CYC6* promoter, when placed in front of a Δ-35 β_2*TUB* minimal promoter, conferred copper responsiveness to the β_2*TUB* promoter. A transfer of heat shock-inducibility from the soybean *Gmhsp17.3-B* promoter to a *CaMV 35S* core promoter has also been demonstrated (Schöffl *et al.*, 1989). Although insertion of up to 300 bp between heat shock elements (HSE) and TATA box have been shown not to interfere with heat shock inducibility (Cohen and Meselson, 1988), our results suggest that heat shock factors bound to the HSEs of the *A* promoter exclusively interact with factors attached to their own, close-by TATA box.

What could be the rationale for the finding that in clones harbouring constructs *AR*, *ARR*, *ARRR*, *ARA*, *ARRA* and *A*β_2*TUB* under basal conditions and after dark-to-light shift only such transcripts were detected that were initiated at the promoter proximal to the *HSP70B-tag* gene? Two possibilities are envisioned that could account for this finding. (i) Transcription initiation is restricted to the promoter located proximal to the *HSP70B* gene. (ii) Due to the presence of additional 5′ sequences, transcripts initiated at (a) distal promoter(s) were unstable or subjected to processing. The latter possibility seems unlikely, since high transcript levels originating from the distal *A* promoter were detected upon heat shock. To account for the first possibility, we speculate that cis-acting sites within the *A* promoter and within the *HSP70B* gene interact with each other via bound proteins: the site(s) within the *A* promoter is thought to constitute a transcriptional enhancer that mediates the observed increase in basal expression and the high level expression upon dark-to-light shift. By promoter deletion analysis, a site fitting this assumption has previously been mapped between nucleotides –108 and –146 (Kropat *et al.*, 1995). Furthermore, a strong binding of nuclear protein(s) to this region has been observed (E.D. von Gromoff and C.F. Beck, unpublished data). We expect that the cis-acting site within the *HSP70B* gene will be situated at the beginning of the gene. These sites may interact via their bound proteins, directing the formation of the transcription initiation complex to the TATA box lying proximal to the *HSP70B* reporter gene. Upon dark-to-light shift, interaction of these sites may be enhanced by additional factor(s), leading to increased transcriptional initiation. Another site located within the *R* promoter is postulated to positively affect both the interaction between the sites described above and the heat shock-induced transcription from the *A* promoter, thus accounting for the synergistic effect observed with *AR* fusions both during dark-to-light shift and heat shock (Figure 3b). Experiments are under way to test the existence of the sites postulated.

In order to determine whether the *AR* promoter fusion may also confer a high level of expression to other genes, we fused the *AR* promoter to the eubacterial *aadA* gene (Prentki and Krisch, 1984). In the case of the *AR-aadA* construct, the *A* promoter appeared to function largely autonomously under inducing conditions. Here, the only well-defined transcripts were those originating from the *A* promoter after light or heat shock induction, which may indicate that the *R* promoter was non-functional or that transcripts originating from the *R* promoter were intrinsically unstable, as was suggested previously (1997b; Cerutti *et al*., 1997a). We speculate that the presence of 67 bp of *HSP70A* 5′ UTR and/or 200 bp of *RBCS2* promoter sequences significantly increased *aadA* transcript stability. Interestingly, the *R-aadA* and *AR-aadA* constructs could be used as selection markers for transformation (Table 1), even though the transcript levels appeared extremely low under the conditions used for selection (continuous light). The fact that 2.5-fold higher transformation rates were obtained for *AR-aadA* as compared to *R-aadA* may also indicate that the presence of the *A* promoter increases the probability that an integrated transgene is expressed. We are currently addressing this question using other reporter genes.

It is not clear whether the transcripts containing promoter sequences at their 5′ ends are translated. According to the scanning model, translation in eukaryotes usually initiates at the first AUG codon following the cap (Kozak, 1987). However, translation initiation also depends on the context around the AUG codon (Kozak, 1986; Kozak, 1997). Although the transcribed Δ-183 *RBCS2* promoter sequence contains several AUGs, neither of these has more than 5–6 matches with the 10 bases comprising consensus sequence for translation initiation in *C. reinhardtii* (Silflow, 1998). In contrast, the context of the true initiation codon of *HSP70B* exhibits 10, that of 9 *aad*A matches.

For transformants expressing *HSP70B* from the *AR* promoter (pCB740), up to twofold higher levels in *HSP70B* transcript and protein have been observed and this property has proved stable for more than 2.5 years (Schroda *et al*., 1999 and data not shown). In contrast, a decrease in expression a few weeks after transformation has been observed in transformants overexpressing the α-tubulin gene under the control of the *R* promoter alone (Kozminski *et al*., 1993). The *AR* promoter as such has already been used successfully by other laboratories to drive high level expression of genomic *Chlamydomonas* genes *CPH1* (*Chlamydomonas* photolyase homologue 1, G.D. Small, personal communication) and *GSP1* (gamete specific protein 1, W.J. Snell, personal communication), and of a synthetic gene coding for a Ble-GFP fusion protein (P. Hegemann, personal communication). Thus, the properties of the *HSP70A* promoter, i.e. inducible high level expression as well as transcriptional stimulation of other promoters, may make it a valuable tool for the further development of *Chlamydomonas* as a model organism.

Experimental procedures

Algal strains and culture conditions

C. reinhardtii strains 302 ($cw_{d,}$ *mt*$^{+}$, *arg*7), 074 ($cw_{d,}$ *mt*$^{-}$, *arg*7) and 106 (*mt*$^{-}$, *arg*7) were kindly provided by R. Matagne (University of Liège, Belgium), strain CC-124 (*mt*$^{-}$) was obtained from the *Chlamydomonas* Culture Collection at Duke University, USA. Strains were grown photomixotrophically in TAP medium (Harris, 1989) on a rotatory shaker at 23°C under continuous irradiation with white light (40 μE $m^{-2}s^{-1}$) provided by fluorescent tubes (Osram L36W/25). TAP medium was supplemented with 100 mg l^{-1} of arginine when required. Light induction and heat shock were performed according to Kropat *et al*. (1995) and von Gromoff *et al*. (1989), respectively. High efficiency mating strains CC-620 and CC-621 were used for the preparation of autolysin (Harris, 1989). Strains CF97 and CF98 were generated by transformation of strains 074 and 106 with pCB412 (G. Glöckner and C.F. Beck, unpublished data), which contains the *ARG7* gene. CF184 and CF185 have been described previously (Schroda *et al*., 1999).

Construction of plasmid vectors

A detailed description of the construction of the plasmids used in this study is available from the web at: http://www.biologie.uni-freiburg.de/data/beck/start.htm/.

Nuclear transformation of C. reinhardtii

C. reinhardtii nuclear transformation was performed using the glass beads method (Kindle, 1990). Prior to transformation, all constructs containing the *ARG7* gene were linearized with *Eco*RI; those with *aadA* or *ARS* fusions were linearized with *Kpn*I. For the transformation of 1×10^8 cells, 100 ng of plasmid DNA was used for *ARG7*-containing plasmids, 500 ng for the others, resulting in a 1 : 5 mass ratio of *ARG7* vector to co-transformed plasmid in co-transformation experiments. The cell wall of strains 074 (not completely cell wall-deficient), 106 and CC-124 was removed by autolysin treatment prior to transformation. Immediately after vortexing with glass beads, cells were spread onto TAP-agar plates, containing 90 μg ml^{-1} spectinomycin (Sigma) for *aadA* constructs. Plates were incubated at 23°C in the light (about 60 μE m^{-2} s^{-1}) and transformants were counted after 2–3 weeks.

Northern blot analyses

For RNA dot blot analyses, transformants were grown in 4 ml TAP medium for 3 days close to the stationary phase. RNA was isolated using acidic phenol as described previously (Schroda *et al*., 1999). After DNaseI treatment (Gibco), approximately 20 μg of RNA were dot-blotted onto nylon membranes (Hybond N, Amersham) according to Sambrook *et al*. (1989).

Large-scale RNA isolation and RNA gel blots were performed as described previously (Schroda *et al*., 1999), except that gels were run for at least 13 h at 50 V. An RNA ladder (Gibco) was used as molecular mass standard.

Radioactive DNA probes were prepared by the random priming technique (Feinberg and Vogelstein, 1983), using [α-^{32}P]dCTP

(Amersham). Blots were probed with the 213 bp tag sequence (detection of the *HSP70B* reporter gene) (Schroda *et al.*, 1999), the 810 bp *Nco*I–*Pst*I fragment containing the *aadA* coding region (Zerges and Rochaix, 1994), two *Bam*HI fragments, 750 bp and 1150 bp in size, from the *ARS* cDNA (Davies *et al.*, 1992), a 370 bp *Sst*II–*Alw*NI fragment from the *RBCS2* coding region that hybridizes to both the *RBCS1* and *RBCS2* mRNAs (Goldschmidt-Clermont and Rahire, 1986), and a 206 bp *Bam*HI–*Nhe*I fragment containing the *RBCS2* promoter (–183 to +15) from pCB738. Hybridization and washing of membranes was carried out as described by Schroda *et al.* (1999). Northern blots were quantitated by exposing membranes to BAS-MP imaging plates (Fuji) and evaluated using the TINA program (Version 2.08d, Raytest). Obtained signals were normalized by the respective *RBCS* signals.

Western blot analysis

SDS-PAGE and transfer of proteins to nitrocellulose membranes (Hybond C-super, Amersham) by semi-dry blotting were carried out as described previously (Schroda *et al.*, 1999). Immunodetection was performed by enhanced chemiluminescence according to the manufacturer's recommendations (Amersham). Quantitation of immunoblots was carried out by the AIDA software package (Version 2.0 beta, Raytest). Antibodies against the HSP70B protein have been described previously (Schroda *et al.*, 1999). Antibodies against the hexahistidine tag were purchased from Dianova (Germany).

Arylsulfatase enzyme activity assays

The selection of co-transformants expressing arylsulfatase was achieved by streaking transformants onto a nylon membrane placed on top of a TAP agar plate and spraying a solution of 5 mM 5-bromo-4-chloro-3-indolyl sulfate (X-SO_4), 10 mM Tris–HCl, pH 7.5, under the lifted membrane (Davies *et al.*, 1992). ARS-expressing clones became blue after 1–24 h. ARS enzyme activity in liquid cultures was measured according to Ohresser *et al.* (1997). Enzyme activity was standardized to total cellular protein of the respective sample. For the determination of the protein concentration, 1 ml of cells was pelleted and solubilized in 0.1 M Na_2CO_3, 0.1 M DTT, 2% SDS. Protein concentrations were determined by staining with amidoblack (Popov *et al.*, 1975). BSA was used as a standard.

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