Reporter genes and highly regulated promoters as tools for transformation experiments in *Volvox carteri*

(Green algae/chimeric genes/arylsulfatase/sex pheromone/extracellular glycoprotein ISG)

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**ABSTRACT** The multicellular alga *Volvox* is an attractive model for the study of developmental processes. With the recent report of successful transformation, regulated promoters as well as reporter genes working in this organism are now required. The *Volvox* genes encoding arylsulfatase and the extracellular glycoprotein ISG are strictly regulated. The former is transcribed only under conditions of sulfur starvation, whereas the latter operates under extreme developmental control—i.e., it is transcribed for only a few minutes in *Volvox* embryos at the stage of embryonic inversion. The gene encoding the sexual pheromone of *Volvox carteri* was placed under the control of the arylsulfatase promoter. In response to sulfur deprivation, *V. carteri* transformed by this construct synthesized and secreted biologically active pheromone. In addition, the gene encoding *Volvox* arylsulfatase was placed under the control of the ISG promoter. Transformed algae synthesized arylsulfatase mRNA only during embryonic inversion. These experiments demonstrate the usefulness of both the arylsulfatase and the sexual pheromone reporter genes. In addition, the highly regulated arylsulfatase promoter allows the construction of inducible expression vectors for cloned genes.

Members of the genus *Volvox* are multicellular photosynthetic organisms and each individual is composed of only two cell types: mortal somatic cells and immortal reproductive cells. In addition, these haploid green algae are capable of both sexual and asexual reproduction. Its simple developmental program makes *Volvox* an attractive model system for studying the mechanism of cellular determination and differentiation by physiological, biochemical, and genetic investigations (reviewed in refs. 1 and 2). A number of genes putatively involved in the control of developmental processes have been cloned (3–6), and a large number of developmental mutants have been described (7–9). However, a serious drawback for molecular biological investigations in this organism was the lack of a reliable technique for genetic transformation. Recently, a *Volvox* transformation method was established by using a helium-flow device to bombard cells with DNA-coated gold particles (10). To allow the application of efficient molecular genetic approaches, reporter genes as well as appropriate promoters working in this organism are now required.

A strong candidate for a homologous reporter gene in *Volvox* is the arylsulfatase gene (11). *Volvox* arylsulfatase is a periplasmic enzyme that cleaves sulfate from aromatic compounds and is therefore readily assayed with chromogenic substrates. Because this enzyme is stable in detergents, it is readily assayed even in crude cell lysates. Furthermore, the enzyme is expressed only under sulfur starvation and no activity is detectable in cells grown in standard sulfate-containing medium. Since the arylsulfatase production is regulated at the transcriptional level (11), the promoter of the arylsulfatase gene is likely to provide a tool for producing inducible expression vectors for cloned genes.

Another highly regulated promoter, which might be useful as an alternative to the inducible arylsulfatase promoter, is provided by the gene encoding the extracellular glycoprotein ISG. This gene, which is developmentally regulated, is transcribed only for about 10 min in the 48-hr life cycle of *Volvox carteri* (3, 12).

The sexual pheromone of *V. carteri* is an attractive alternative to the arylsulfatase as a reporter gene. This sex-inducing pheromone is a glycoprotein synthesized and released by sperm cells of male strains (4–7, 13–15). Extremely low concentrations of the pheromone convert asexually growing males and females to the sexual pathway. This allows sensitive detection of gene expression, as sexually induced algae are easily identified by inspection with a stereo microscope.

In this paper we describe two reporter gene systems in *V. carteri*—the sexual pheromone expressed under the control of the arylsulfatase promoter and arylsulfatase expressed under the control of the ISG promoter—and thereby show that these promoters and reporter genes can serve as tools for transformation experiments in *Volvox*.

**MATERIALS AND METHODS**

**Recipient Strain.** The strain 153-48 used as DNA recipient is an F1 female progeny of HB11A, a female strain of *Volvox carteri* f. nagariensis that has previously been described (16). This strain—with wild-type morphology inherited from HB11A, a stable mutant allele that confers resistance to chloride—has lost the ability to utilize nitrate as a nitrogen source and is therefore inferred to be the result of a stable loss-of-function mutation of *nitA*, the structural gene encoding nitrate reductase (17). Strain 153-48 was obtained from R. Schmitt, University of Regensburg.

**Culture Conditions.** Synchronous *Volvox* recipients were grown in *Volvox* medium (18) at 28°C in an 8-hr dark/16-hr light (10,000 lx) cycle (14). The nonselective medium used was *Volvox* medium supplemented with 1 mM NH₄Cl; selective medium was *Volvox* medium lacking NH₄Cl, containing only nitrate as a nitrogen source. In *Volvox* medium lacking sulfate, MgSO₄ was replaced by MgCl₂.

**Gene Splicing by Overlap Extension (Gene SOEing).** The gene SOEing technique was performed as described by Horton et al. (19). To construct the arylsulfatase-sexual pheromone hybrid DNA fragment, the first polymerase chain reaction (PCR) was performed on arylsulfatase A phase clone λ5/2 (11) with a 20-mer 5’ sense primer (5’TTCGCCACCTTCCGACCGACG-3’) and a 50-mer 3’ antisense primer (5’CAGAAATTTGACTACACTACTGCGACCCGACATGGTGTTGTCC-3’) with only half of the primer

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matching. The second PCR was performed on sexual pheromone \( \lambda \) clone Ind-28 (5) with a 20-mer 3' antisense primer (5'-ACAGCCATGAAGACCCCTGGC-3') and a 50-mer 5' sense primer (5'-GGACAAACCAGTCGGCCTGACGGATGGCAGATGTGGTATGCTAACATTCTG-3'), again with only half of the primer matching. The third PCR was carried out with both these PCR products and only the flanking primers. To construct the ISG/arylsulfatase hybrid DNA fragment, exactly the same strategy was applied. Therefore, the first PCR was performed with a genomic \( \lambda \) clone encoding ISG (3), a 19-mer 5' sense primer (5'-GACGAAGCGCTTGGATCC-3') and a 34-mer 5' sense primer (5'-CGACCAATCGTTGCAACATCCTAACCCAAACGGC-3'). The second PCR was performed with the arylsulfatase clone \( \lambda \)5/2 (11), a 17-mer 3' antisense primer (5'-TCTTGAAGCTCAATGCCG-3') and a 34-mer 5' sense primer (5'-GCGCTTTGGGATGATTGTTGCAAGCTTGTGTCG-3'). The third PCR was performed in the presence of both these PCR products and the flanking primers only. PCRs were carried out in PCR buffer (50 mM Tris-HCl, pH 8.5/50 mM NaCl/2.5 mM MgCl2/2 mM dithiothreitol) containing 50 pmol of sense and antisense primers. Thirty cycles of PCR amplification (94°C, 45 sec; 50°C, 30 sec; 72°C, 40 sec) were performed. 

**Cloning.** The final construction of the arylsulfatase/sexual pheromone chimeric gene containing a 2.3-kb fragment of the arylsulfatase 5' region derived from arylsulfatase clone \( \lambda \)5/2 (11) and the coding region for the sexual pheromone (3.6 kb) derived from \( \lambda \) clone Ind-28 (5) was performed by standard techniques (20), yielding a plasmid designated pArInK2/1. The ISG/aryl-sulfatase chimeric gene was generated by using a 2.9-kb fragment of the 5' region derived from the ISG genomic \( \lambda \) clone (3) that was fused to a 10.0-kb fragment containing the coding region of the arylsulfatase genomic sequence (11) in pUC18, yielding a plasmid designated pISG-Ark2/40. In both cases, the fusion regions were generated by the gene SOEing technique. Products of cloning were sequenced by the chain-termination method (21).

**Preparation of Plasmid DNA.** Plasmid DNAs were prepared as described by Hattori and Sakaki (22). Final purification of the plasmids was achieved by CsCl density gradient centrifugation (20).

**Transformation.** *V. carteri* strain 153-48 was transformed by using flowing helium to bombard cells with DNA-coated gold particles (10, 23). Plasmid pArInK2/1, carrying the sexual pheromone coding sequence under the control of the arylsulfatase promoter, and plasmid pISG-Ark2/40, carrying the arylsulfatase coding sequence under the control of the ISG promoter, were each introduced into *V. carteri* strain 153-48 by cotransformation with plasmid pVcNR1 (10, 24). Plasmid pVcNR1 contains 5.8 kb of the coding region of the *V. carteri* nitA gene plus ~1 kb of downstream and ~1 kb of upstream DNA in the vector pBS(+) (Stratagene) (24) and was therefore used to complement the stable nitA mutation in *V. carteri* strain 153-48.

Particles for transformation were prepared for use by mixing the plasmids and a sterile aqueous suspension of gold particles (1-3 \( \mu \)m in diameter; Aldrich) in the desired proportions. Then, 0.1 volume of 3 M NaOAc and 2.5 volumes of ethanol were added, and after incubation at -20°C for at least 1 hr, particles were collected by centrifugation, washed once with 70% ethanol, and finally resuspended in ethanol. Recipient cultures of strain 153-48 were collected on a 100-\( \mu \)m nylon screen shortly before or after the onset of embryonic cleavages. The spheroids were broken up by forcing them through a 0.5-mm hypodermic needle. The cell suspension was filtered through a 100-\( \mu \)m nylon screen through which only free gonidia, free embryos, and single somatic cells can pass. Gonidia and embryos were then collected by low-speed centrifugation. The centrifugation step was repeated several times and the supernatant, which contained some somatic cell sheets, was discarded each time and replaced with fresh medium. The resulting target cells were resuspended in selective *Volvox* medium lacking NH4Cl. They were then bombarded by the method of Takeuchi et al. (23). Bombarded cultures were observed from the sixth day after transformation on, and each green organism was transformed into a microtiter well with fresh selective *Volvox* medium. Putative transformants were tested for restoration of chlorate sensitivity by cultivation in 8 mM potassium chlorate in *Volvox* medium (10).

**Assay of Sexual Pheromone Activity.** *Volvox* transformants were collected on a nylon screen. After extensive washing with *Volvox* medium lacking sulfate, the algae were incubated in sulfate-free medium (500 \( \mu \)l) under standard conditions for 9 hr in microtiter wells. At the end of this period, sulfate was added and the algae were further incubated. Alternatively, the sulfur-starved algae were removed and 0.1 volume of the culture medium was added to algae grown in sulfur-sufficient medium. Sexually induced algae were indentified by inspection under a stereo microscope.

**Assay of Arylsulfatase Activity.** *Volvox* transformants were cultivated in 500 \( \mu \)l in microtiter wells. Four microliters of 50 mM 5-bromo-4-chloro-3-indoly sulfate was added and cultures were further incubated at 28°C. Algae expressing arylsulfatase were identified by their blue color.

PCR with DNA from 20 *Volvox* spheroids. Twenty *Volvox* spheroids after their release from the mother spheroid were selected under a stereo microscope and transferred into 20 \( \mu \)l of sterile lysis buffer (50 mM Tris-HCl, pH 8.0/300 mM NaCl/5 mM EGTA/2% SDS). After 10 min at 30°C, *Volvox* spheroids were removed under the sterile microscope and RNA was precipitated with 60 \( \mu \)l of ethanol. The precipitate was washed in 70% ethanol and dissolved in 10 \( \mu \)l of reverse transcriptase buffer [50 mM Tris-HCl, pH 8.3/40 mM KCl/6 mM MgCl2/1 mM dithiothreitol/1 mM dNTPs with RNAguard (Pharmacia) at 1 unit/\( \mu \)l]. Antisense primers 5'-CGTGCAGAGCCCAA-CAG-3' (sexual pheromone) and 5'-GGATGCGTTGAGT-TCTG-3' (aryl-sulfatase) (50 pmol of each) were added to DNA derived from transformants containing the arylsulfatase promoter/sexual pheromone chimeric gene. Antisense primers 5'-GGATGCGTTGAGTCAG-3' (aryl-sulfatase) and 5'-GGGAAATAGCTGTTCC-3' (ISG) (50 pmol of each) were added to DNA derived from transformants containing the ISG promoter/aryl-sulfatase chimeric gene. Reverse transcription was carried out with 100 units of Moloney murine leukemia virus reverse transcriptase (Pharmacia) for 60 min at 40°C; then 90 \( \mu \)l of PCR buffer containing 100 pmol of sense primer (5'-TGCTGCAACTGCGAGG-3', for transformants containing the arylsulfatase promoter/sexual pheromone chimeric gene, or 5'-TAATAGTAAACCTGATCC-3', for transformants containing the ISG promoter/aryl-sulfatase chimeric gene) was added, and 38 cycles of PCR amplification (94°C, 45 sec; 54°C, 30 sec; 72°C, 45 sec) were performed. Products of PCR amplification were ligated into the *Sma* I site of pUC18 and sequenced by the chain-termination method (21).

**RESULTS AND DISCUSSION**

**Arylsulfatase Promoter/Sexual Pheromone Construct.** *V. carteri* genomic clones encoding arylsulfatase (11) and the sexual pheromone (5) were used to construct a chimeric gene consisting of the promoter region of the arylsulfatase gene and the coding region of the sexual pheromone gene. The fusion region (between the *Xho* I and *Apa* I restriction sites) was generated by the gene SOEing technique (19). The general mechanism is illustrated in Fig. 1. The gene SOEing technique is a fast and extremely powerful way of recombining and modifying nucleotide sequences. The complete construct, pArInK2/1, is shown in Fig. 2A. The arylsulfatase
The ISG part of the construct starts 2.9 kb upstream of the start codon and ends with the base preceding the ATG start codon of the ISG coding sequence. The arylsulfatase part starts with the ATG start codon of the arylsulfatase sequence and ends 2.2 kb downstream of the stop codon.

**Transformation.** For transformation, flowing helium was used to bombard the reproductive cells with DNA-coated gold particles (23). Plasmids pArInK2/1 and pISGArK2/40 were each introduced into the V. carteri nitrate-utilizing mutant strain 153-48 by cotransformation with pVcNR1 (10, 24). The latter plasmid contains the nitrate reductase gene, which complements the loss-of-function mutation of nitA and enables transformants of 153-48 to grow on medium containing nitrate as a sole nitrogen source (10).

**Sexual Pheromone Activity in Response to Sulfur Deprivation.** To identify cotransformants containing the arylsulfatase promoter/sexual pheromone chimeric gene, transformants were incubated in sulfate-free medium for 9 hr. After that starvation period, the algae were further incubated in growth medium containing sulfate. Expression of the sexual pheromone under the control of the arylsulfatase promoter would result in self-induction of the female strain. Although some stress situations [heat shock, aldehyde treatment, protease digestion of matrix, etc. (reviewed in ref. 2)] can induce pheromone production, sulfate deprivation does not cause any self-induction in the female recipient strain 153-48 (data not shown). Therefore, we looked for sexually induced transformants, which are easily recognized by microscopic inspection. Five self-induced female transformants were identified and one of them, T1/44/2, was further characterized. This clone produces gonidia and reproduces asexually in sulfate-containing medium (Fig. 3A) but produces egg-bearing sexual females after having been exposed to sulfate-free medium for 9 hr (Fig. 3B). The sexual pheromone produced under these conditions is secreted into the culture medium, as aliquots of this medium are able to induce sexual development of female wild-type strain HK10 or male wild-type strain 69-1b.

**Arylsulfatase Synthesis Under Developmental Control.** To identify cotransformants containing the ISG promoter/aryl sulfatase chimeric construct, the chromogenic substrate

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**Fig. 1.** Construction of the overlap region in chimeric gene constructs by gene SOEing. The segments to be joined (fragment a-b from gene I and fragment c-d from gene II) were amplified in separate PCRs (PCRs 1 and 2). Primers b and c are complementary to one another; half of their nucleotide sequence is derived from gene I and the other half from gene II. This causes both PCR products to share homologous sequences at the ends to be joined. The presence of both these products and the primers a and d in the third PCR mixture allows the amplification of the recombiant product. The PCR product is digested with the restriction enzymes RE 1 and RE 2 as indicated and cloned as described in the text.

**ISG Promoter/Arylsulfatase Construct.** The chimeric gene consisting of the promoter region of the ISG gene (3) and the coding region of the arylsulfatase gene (11) was constructed in the same manner by using the corresponding genomic clones. Again, the fusion region (between the BamHI and EcoRI restriction sites) was generated by the gene SOEing technique (Fig. 1). The resulting construct, pISGArK2/40, is shown in Fig. 2B. The ISG part of the construct starts 2.9 kb upstream of the start codon and ends with the base preceding the ATG start codon of the ISG coding sequence. The arylsulfatase part starts with the ATG start codon of the arylsulfatase sequence and ends 2.2 kb downstream of the stop codon.

**Fig. 2.** Structure of chimeric genes. (A) Structure of the arylsulfatase/sexual pheromone chimeric gene of plasmid pArInK2/1, containing the arylsulfatase promoter region and the sexual pheromone coding region. (B) Structure of the ISG/aryl sulfatase chimeric gene of plasmid pISGArK2/40, containing the ISG promoter region and the arylsulfatase coding region. Intron/exon structure is given below the physical map. Gene SOEing was performed as described in the text to create the fusion region. tsp, Transcription start point.
5-bromo-4-chloro-3-indolyl sulfate was added to the sulfate-containing growth medium to allow easy detection of algae expressing arylsulfatase by their blue color. Since wild-type arylsulfatase is expressed only in sulfate-free medium, any detected arylsulfatase activity identifies a cotransformant. Several cotransformants were obtained, and one of them, T3/5/2, was further characterized. When the chromogenic substrate 5-bromo-4-chloro-3-indolyl sulfate was added to isolated embryos from this clone, arylsulfatase activity could be detected immediately after the inversion process was completed (Fig. 4).

**PCR Amplification of the Chimeric Transcripts.** Reverse transcription–PCR was used to verify the existence of hybrid mRNA in transformants and both the degree of expression and regulation. For this purpose, RNA was extracted from 20 *Volvox* spheroids, reverse transcribed, and subsequently amplified by PCR. Oligonucleotide primers were selected that allowed amplification of the fusion region of the corresponding chimeric construct. To determine the ratio of hybrid mRNA and the corresponding endogenous mRNA, cDNAs derived from both transcripts were simultaneously amplified in the same reaction tube. Referring to clone T1/44/2 containing the arylsulfatase promoter/sexual pheromone construct, a 200-bp and a 147-bp PCR product were predicted to be recovered if both the endogenous gene and the transformed gene were transcribed and processed properly. Likewise, for clone T3/5/2 containing the ISG promoter/aryl sulfatase construct, a 256-bp and a 399-bp PCR product were expected. These predictions were made from the sequence data and the known intron/exon boundaries of the parent genes.

Accumulation of mRNA derived from the arylsulfatase promoter/sexual pheromone construct was analyzed after growth of clone T1/44/2 in sulfate-free medium for various times. RNA was extracted from transformants after sulfate starvation for 1, 3, and 6 hr. Reverse transcription and subsequent PCR amplification yielded both the expected chimeric cDNA and the endogenous arylsulfatase cDNA in a ratio of ≈1:1. Both cDNAs appeared as early as 3 hr after the initiation of sulfate deprivation (Fig. 5A). mRNA levels reached a maximum 6 hr after the initiation of sulfate deprivation. DNA sequence analysis of the chimeric cDNA fragment (147 bp) is shown in Fig. 6A. All introns within the construct were excised by the splicing machinery of *Volvox* exactly in the same manner as known for the parent genes. Thus, the arylsulfatase promoter allowed the strictly controlled expression of a gene.

The same strategy was applied to demonstrate the production of hybrid mRNA derived from the ISG promoter/aryl sulfatase construct in clone T3/5/2 (Fig. 5B). Reverse transcription and subsequent PCR amplification of both the chimeric cDNA and the endogenous ISG cDNA yielded the two fragments in a ratio of ≈1:1. Both mRNA species were detectable only during the short period of embryonic inversion (Fig. 5B). Therefore, the ISG promoter fragment used was able to mediate this extreme type of developmental control of gene expression. The result of a DNA sequence analysis of the chimeric cDNA fragment (399 bp) is shown in Fig. 5B. Again, all introns were correctly excised in the manner known for the parent genes. This particular construct should allow a detailed analysis of this ISG promoter system by deletion analysis and site-directed mutagenesis. At the end of the experiment, both the chimeric cDNA and the endogenous ISG cDNA in one reaction tube yielded 147-bp (derived from the chimeric gene) and 200-bp (derived from the endogenous arylsulfatase gene) DNA fragments in a ratio of ≈1:1 as early as 3 hr after the initiation of sulfate deprivation. (B) RNA was extracted from 20 *Volvox* embryos of the transformant T3/5/2, containing the ISG/aryl sulfatase hybrid DNA, 5 hr (lane 1) or 1 hr (lane 2) prior to inversion, during inversion (lane 3), and 1 hr after inversion (lane 4). Reverse transcription and subsequent PCR amplification of both the chimeric cDNA and the corresponding endogenous ISG cDNA in one reaction tube yielded 399-bp (chimeric gene) and 256-bp (endogenous gene) DNA fragments in a ratio of ≈1:1 only during the inversion process. Sizes of the PCR products were determined by using a 123-bp ladder as size marker and by DNA sequencing.
Fig. 6. DNA sequences from reverse transcription and subsequent PCR amplification of the fusion region of the ISG/arylsulfatase gene from clone T1/44/2 (A) and the fusion region of the ISG/arylsulfatase gene from clone T3/5/2 (B). Positions of introns are indicated by vertical arrowheads. An asterisk denotes the last base contributed by the arylosulfatase or ISG 5' sequence; the following base is the first contributed by the structural gene sequence of the sexual pheromone or of the arylosulfatase. In addition, this position is the translation initiation site. PCR primers are represented by horizontal arrows.

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