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The *Chlorella* hexose/H^+ symporter is a useful selectable marker and biochemical reagent when expressed in *Volvox*

(green algae/recombinant DNA technology/plant transformation/heterologous expression/cotransporter)

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ABSTRACT **The multicellular obligately photoautotrophic alga *Volvox* is composed of only two types of cells, somatic and reproductive. Therefore, *Volvox* provides the simplest model system for the study of multicellularity. Metabolic labeling experiments using radioactive precursors are crucial for the detection of stage- and cell-type-specific proteins, glycoproteins, lipids, and carbohydrates. However, wild-type *Volvox* lacks import systems for sugars or amino acids. To circumvent this problem, the hexose/H^+ symporter (*HUP1*) gene from the unicellular alga *Chlorella* was placed under the control of the constitutive *Volvox* β-tubulin promoter. The corresponding transgenic *Volvox* strain synthesized the sugar transporter in a functional state and was able to efficiently incorporate ^{14}C from labeled glucose or glucosamine. Sensitivity toward the toxic glucose/mannose analogue 2-deoxyglucose increased by orders of magnitude in transformants. Thus we report the successful transformation of *Volvox* with a gene of heterologous origin. The chimeric gene may be selected for in either a positive or a negative manner, because transformants exhibit both prolonged survival in the dark in the presence of glucose and greatly increased sensitivity to the toxic sugar 2-deoxyglucose. The former trait may make the gene useful as a dominant selectable marker for use in transformation studies, whereas the latter trait may make it useful in development of a gene-targeting system.**

The transition from unicellularity to multicellularity can be analyzed in a family of organisms collectively referred to as the Volvocaceae. The simpler organisms among the volvocaceans represent aggregates of *Chlamydomonas*-like cells, whereas more advanced members of this group, like *Volvox*, have developed two cell types with a complete division of labor. In *Volvox carteri*, about 2000 cells are somatic and only 16 cells are reproductive (1, 2). Thus, *Volvox* represents multicellularity in its simplest form. Biochemical studies of developmental processes in this organism require efficient procedures for metabolic labeling of relevant molecules. However, up to now, efficient techniques for pulse- and pulse–chase-labeling experiments were only available for molecules incorporating sulfate and phosphate. Since *Volvox* lacks transport systems for sugars or amino acids, incorporation of ^{14}C was inefficient and could only be achieved in a rather indirect way using radioactive carbon dioxide. Labeling experiments using radioactive glucose would be most helpful for identifying stage- and cell-type-specific proteins, glycoproteins, lipids, and carbohydrates. Since other green algae, like the conditionally heterotrophic unicellular alga *Chlorella*, are equipped with sugar transport systems, we tried to express a *Chlorella* sugar transporter in *Volvox*. To that end, the hexose/H^+ symporter (*HUP1*) gene from *Chlorella* (3) was placed under the control of the constitutive β-tubulin promoter from *Volvox* (4). The *Chlorella* hexose/H^+ symporter HUP1 exhibits high homology to the human glucose transporter (5) and belongs to a large family of substrate transporters exhibiting 12 transmembrane helices (6).

In this paper, we describe the functional expression of the *Chlorella HUP1* gene in *V. carteri* and show the usefulness of this particular transgenic *Volvox* for biochemical and genetic work. To our knowledge, the *Chlorella HUP1* gene is the first gene of heterologous origin successfully expressed in *Volvox*.

MATERIALS AND METHODS

Recipient Strain. *Volvox* 153-48 was used as DNA recipient. Strain 153-48 is a F_1 female progeny of HB11A, a female strain of *Volvox carteri* f. *nagariensis* that has been described (7). Strain 153-48 with wild-type morphology inherited an allele of HB11A that confers resistance to chlorate, abolishes 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 (8).

Culture Conditions. Synchronous *Volvox* recipients were grown in *Volvox* medium (9) at 28°C in an 8-h dark/16-h light (10,000 lux) cycle (10). The nonselective medium used was *Volvox* medium, supplemented with 1 mM NH_4Cl; selective medium was *Volvox* medium lacking NH_4Cl and containing only nitrate as a nitrogen source.

Cloning. An ≈2.1-kb *Sal* I–*Dra* I fragment of the *Volvox* β-tubulin 5′ region (4) was cloned into a *Sal* I/*Eco*RV-digested pIC20H vector (11), resulting in clone pIK1. Construction of the *Volvox* β-tubulin–*Chlorella HUP1* chimeric gene containing the ≈2.1-kb fragment of the *Volvox* β-tubulin gene derived from clone pIK1 and the coding region for *HUP1* derived from clone pLM100 (3) was performed by standard techniques (12). The resulting plasmid was designated pβHUP1. Products of cloning were sequenced by the chain-termination method (13).

Preparation of Plasmid DNA. Plasmid DNAs were prepared as described by Hattori and Sakaki (14). Final purification of plasmids was achieved by CsCl density gradient centrifugation (12).

Transformation. *V. carteri* strain 153-48 was transformed by using flowing helium to bombard cells with DNA-coated gold particles (15–17). Plasmid pβHUP1, carrying the *Chlorella HUP1* coding sequence under the control of the *V. carteri* β-tubulin promoter, was introduced into *V. carteri NitA*$^-$ strain 153-48 by cotransformation with plasmid pVcNR1 (8, 16). 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) and was, therefore, used to complement the stable *nitA* mutation in *V. carteri* strain 153-48. Microprojectiles for transformation were prepared with gold particles (1–3 μm in diameter, Aldrich) as described (17). Recipient cultures of strain 153-48 were collected on a 100-μm nylon screen shortly before or after the onset of embryonic cleavages. The spheroids were broken up

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Abbreviation: HU-T, hexose uptake-transformant.

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by forcing them through a 0.5-mm hypodermic needle. The cell suspension was filtered through a 100-μm (pore size) nylon screen through which only free gonidia, free embryos, and single somatic cells can pass. Gonidia/embryos were then collected on a 10-μm nylon screen. The gonidia/embryo suspension was allowed to settle several times and the supernatant, which contains some somatic cell sheets, was discarded each time. The resulting target cells were resuspended in selective *Volvox* medium lacking NH_4Cl. They were then bombarded by the method of Takeuchi *et al.* (15). Bombarded cultures were observed from the sixth day after transformation on, and each green organism was transferred 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 (16).

Assays of [^{14}C]Glucose and [^{14}C]Glucosamine Transport. About 50 *Volvox* spheroids were suspended in 100 μl of standard *Volvox* medium. After addition of 2 μCi (74 kBq) of D-[U-^{14}C]glucose (specific activity, 295 mCi/mmol) or D-[U-^{14}C]glucosamine (specific activity, 212 mCi/mmol), the suspension was incubated at 28°C for 1 h. After the pulse period, the colonies were washed with *Volvox* medium containing 50 mM glucose. Washed algae were disintegrated by ultrasonic treatment (30 sec total). Radioactivity was measured in a liquid scintillation counter (BETAmatic BASIC, Kontron, Zurich). For gel electrophoresis, the lysate was centrifuged at 135,000 *g* for 2 h. The pellet (crude membrane fraction) was dissolved in standard SDS gel loading buffer, heated to 95°C, and subjected to standard SDS/PAGE.

Assay of 2-Deoxyglucose Transport. Algae were grown at various concentrations of the toxic sugar 2-deoxyglucose for 3 days. Transformants, possessing the *Volvox* β-tubulin–*Chlorella HUP1* chimeric gene, were able to import 2-deoxyglucose and were, therefore, killed. Dead algae were identified under the stereo microscope.

Reverse Transcription–PCR with Transformants. Twenty *Volvox* spheroids after their release from the mother spheroid were selected under the stereo microscope and transferred into 20 μl 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 stereo microscope and RNA was precipitated with 60 μl of ethanol (18, 19). The washed precipitate (70% ethanol) was dissolved in 10 μl of reverse transcriptase buffer containing 50 mM Tris·HCl (pH 8.3), 40 mM KCl, 6 mM $MgCl_2$, 1 mM dithiothreitol, 10 units of RNAguard (Pharmacia), and all four dNTPs (each at 1 mM) (19). The antisense primer 5′-GCCTGTATCCCAGCTCA (*HUP1*) (50 pmol) was added to RNA derived from transformants containing the *Volvox* β-tubulin–*Chlorella HUP1* chimeric gene. Reverse transcription was carried out with 100 units of Moloney murine leukemia virus reverse transcriptase (Pharmacia) for 60 min at 40°C and then 90 μl of PCR buffer (50 mM Tris·HCl, pH 8.5/50 mM NaCl/2.5 mM $MgCl_2$/2 mM dithiothreitol) containing 100 pmol of sense primer 5′-ATAACAAGCGACCACTAC (β-tubulin) was added, and 38 cycles of PCR amplification (94°C, 30 sec; 54°C, 20 sec; 72°C, 20 sec) were performed. Products of PCR amplification were ligated into the *Sma* I site of pUC18 and sequenced by the chain-termination method (13).

RESULTS

Construction of the Chimeric Gene. Genomic clones encoding *V. carteri* β-tubulin (4) and *Chlorella HUP1* (3), a monosaccharide/H^+ symporter, were used to construct a chimeric gene consisting of the promoter region of the β-tubulin gene and the coding region of the *Chlorella HUP1* gene. Plasmid pIK1, bearing the 2.1-kb *Volvox* β-tubulin promoter region (4), was partially digested with *Hin*dIII and ligated to a 5.6-kb *Hin*dIII fragment from plasmid pLM100 (3) containing the *HUP1* gene. The pLM100 *Hin*dIII fragment begins 270 bp in front of the start ATG codon of the *HUP1* gene and ends about 800 bp behind the stop codon. It contains 13 introns, one in front of the start ATG. pβHUP1, the complete construct, was confirmed by sequencing. The physical map of pβHUP1 is shown in Fig. 1.

Stable Transformation. For transformation, flowing helium was used to bombard the *Volvox* reproductive cells with DNA-coated gold particles (15–17). Plasmid pβHUP1, containing the *Volvox* β-tubulin promoter–*Chlorella HUP1* gene construct, was introduced into the *V. carteri NitA*$^-$ strain 153-48 by cotransformation with pVcNR1 (8, 16). 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 (16).

Reverse Transcription–PCR Amplification and Sequencing of the Chimeric Transcript. The reverse transcription–PCR technique was used to verify the existence of hybrid mRNA in transformants. 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 chimeric β-tubulin promoter–*Chlorella HUP1* gene construct. From transformants containing the *Volvox* β-tubulin promoter–*Chlorella HUP1* gene construct, a 362-bp PCR product was predicted if the chimeric gene is transcribed and processed properly. This prediction was made from the sequence data and the known intron–exon boundaries of the parent genes.

Reverse transcription and subsequent PCR amplification yielded the expected chimeric 362-bp cDNA in 12 out of 14 transformants. The result of a reverse transcription–PCR using 1 of these 12 hexose uptake-transformants (HU-Ts), named HU-T17, is shown in Fig. 2. The corresponding DNA sequence analysis of this cDNA fragment (362 bp) is shown in Fig. 3.

The intron originally present within this stretch of chimeric DNA was excised by the splicing machinery of *Volvox* exactly as known for the parent *Chlorella HUP1* gene. Thus, the *Volvox*

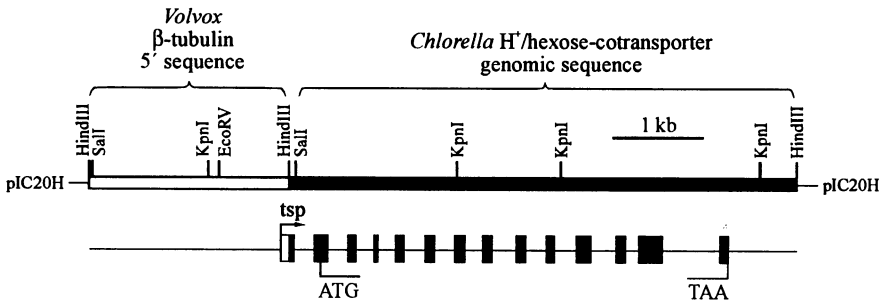

FIG. 1. Structure of the chimeric gene, in plasmid pβHUP1, containing the *Volvox* β-tubulin promoter region and the *Chlorella* hexose/H^+ cotransporter coding region. Intron–exon structure is given below the physical map. pβHUP1 contains 21 bp between the β-tubulin promoter and the *HUP1* gene (see Fig. 3) that are derived from the multiple cloning site of the vector pIC20H. tsp, Transcription start point.

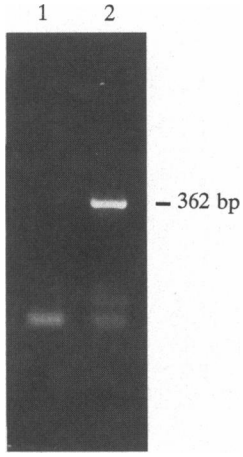

FIG. 2. Reverse transcription and subsequent PCR amplification of the chimeric mRNA. RNA was extracted from 20 *Volvox* spheroids of the parent strain 153-48 (lane 1) or of the transformant HU-T17 (lane 2) containing the *Chlorella* hexose/H^+ symporter gene under the control of *Volvox* β-tubulin promoter. Size of the PCR product was determined by DNA sequencing.

β-tubulin promoter allowed the transcription and processing of a *Chlorella* gene in *Volvox*.

[^{14}C]Glucose Uptake Assay. Transgenic algae containing the intact chimeric construct were tested for their ability to take up [^{14}C]glucose. Therefore, 50 *Volvox* algae were incubated in *Volvox* medium containing D-[U-^{14}C]glucose for 1 h in the dark. Afterward, colonies were washed thoroughly, disintegrated by ultrasonic treatment, and assayed for incorporated radioactivity (Fig. 4).

In most of the transformants, incorporated radioactivity increased up to 300-fold, indicating that the *Chlorella HUP1* sugar transporter was expressed in a functional state.

[^{14}C]Glucose Incorporation. The incorporation of ^{14}C into macromolecules of transformant HU-T17 after [^{14}C]glucose pulse labeling was compared to the parent *NitA*$^-$ strain 153-48. Extracts from labeled algae were loaded on a SDS/polyacrylamide gel and labeled components were visualized by fluorography (Fig. 5*A*). Transformant HU-T17 incorporates ^{14}C into a broad range of macromolecules, whereas no ^{14}C incorporation is detectable in the parent *NitA*$^-$ strain 153-48 (Fig. 5*A*).

Survival in the Dark in Glucose-Containing Medium. Transformants were tested for their ability to grow in the dark in the presence of glucose. Therefore, HU-Ts, wild-type *Volvox* algae (HK10), and the parent *NitA*$^-$ strain (153-48) were incubated in *Volvox* medium containing 40 mM glucose for 14 days in the dark and subsequently in the standard 8-h dark/16-h light cycle. Both transformants and parent strains did not grow during the 14-day dark phase, but 75% of the transformants survived this period and started to grow again, whereas

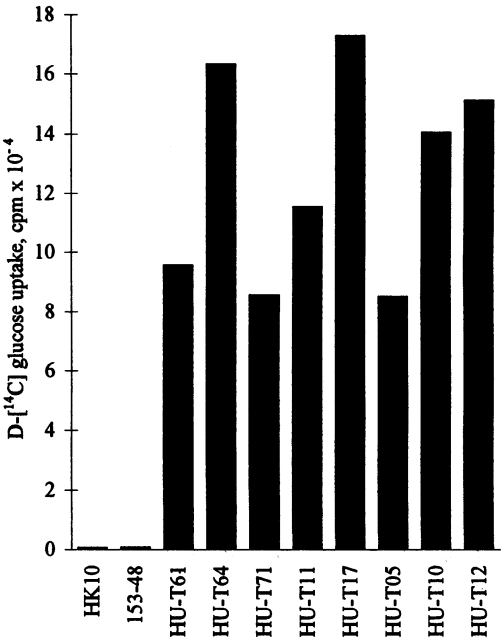

FIG. 4. [^{14}C]Glucose uptake assay. Wild-type *Volvox* (HK10), the parent *NitA*$^-$ strain (153-48), and HU-T transformants were grown in the presence of D-[U-^{14}C]glucose. D-[^{14}C]Glucose uptake is given in cpm per 10 *Volvox* spheroids per 60 min.

all of the wild-type *Volvox* spheroids (HK10) and the parent 153-48 spheroids died.

Effect of 2-Deoxyglucose. 2-Deoxyglucose as an analogue of D-glucose and D-mannose is toxic if it is allowed to pass the cell membrane, probably because of its interference with protein glycosylation (20). Two mechanisms for this inhibitory effect are discussed. Accumulated 2-deoxy-D-glucose 6-phosphate could affect the activity of hexose phosphate isomerase, thus, leading to a shortage of substrates essential for glycosylation (21, 22). Alternatively, glycoprotein biosynthesis may be affected by the incorporation of 2-deoxy-D-glucose into the polysaccharide resulting in an altered carbohydrate moiety (23, 24).

Transformants were tested for their ability to take up 2-deoxyglucose. They were therefore treated with different concentrations of 2-deoxyglucose and the survival rates were compared to wild-type algae under the same conditions. Wild-type algae (HK10) and the parent *NitA*$^-$ strain 153-48 grew at concentrations up to 3×10^{-3} M 2-deoxyglucose without any loss of viability. In contrast, transformants died at concentrations of 2-deoxyglucose as low as 1×10^{-5} M, indicating an increase of sensitivity toward 2-deoxyglucose by orders of magnitude (Fig. 6). Transformed *Volvox* are dead in only 3 days in 2-deoxyglucose-containing medium.

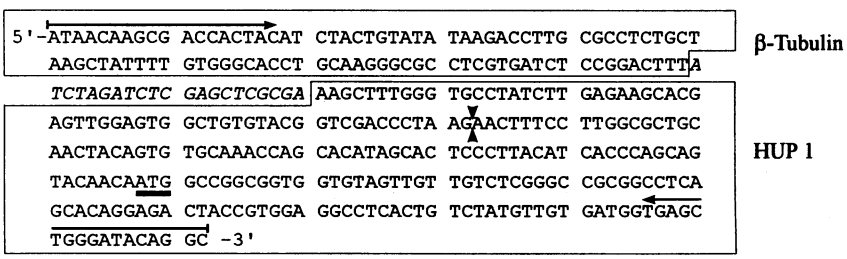

FIG. 3. Sequence of cDNA obtained from reverse transcription and subsequent PCR amplification of the fusion region of the *Volvox* β-tubulin–*Chlorella* hexose/H^+ symporter gene of transformant HU-T17. The position of an intron is indicated by two vertical arrowheads. The nucleotides between the boxed β-tubulin 5′ sequence and the boxed sequence of the *Chlorella* hexose/H^+ cotransporter gene fragment are derived from the cloning site of the vector pIC20H and are shown in italic type. The translation initiation site is underlined. PCR primers used are indicated by horizontal arrows.

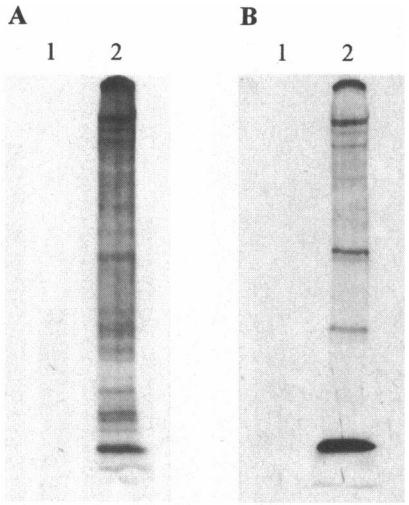

FIG. 5. Comparison of ^{14}C incorporation into macromolecules of the parent *NitA*$^-$ strain (153-48) and of transformant HU-T17 after pulse labeling. Fluorogram of a SDS/polyacrylamide gel (4–15% gradient). (*A*) Incorporation of D-[^{14}C]glucose into macromolecules of parent strain 153-48 (lane 1) and of transformant HU-T17 (lane 2). X-ray film after a 0.5-day exposure. (*B*) Incorporation of D-[^{14}C]glucosamine into macromolecules of parent strain 153-48 (lane 1) and of transformant HU-T17 (lane 2). X-ray film after a 3-day exposure.

[^{14}C]Glucosamine Uptake and Incorporation. [^{14}C]Glucosamine labeling is attractive for biochemical work, because this particular sugar becomes incorporated into glycoproteins and glycosyl-phosphatidylinositol anchors if it gets into eukaryotic cells. As the HUP1 transporter is known to be able to transport glucosamine in addition to glucose, transformants were checked for the uptake of [^{14}C]glucosamine. Again, 50 *Volvox* spheroids were pulse-labeled as described for [^{14}C]glucose. Labeled spheroids were washed and disintegrated, and radioactivity was measured (Fig. 7). In transformants a 100-fold increase of incorporated radioactivity was measured, indicating the active transport of [^{14}C]glucosamine into the cells.

The incorporation of [^{14}C]glucosamine into biopolymers of transformant HU-T17 was confirmed by SDS/polyacrylamide gel electrophoresis and fluorography. The results are shown in Fig. 5*B*. No incorporation is found in the parent *NitA*$^-$ strain 153-48. In comparison with [^{14}C]glucose labeling, the degree of ^{14}C incorporation into macromolecules of transformant HU-T17 is much lower and more selective after labeling with [^{14}C]glucosamine, although both sugars are taken up at comparable rates.

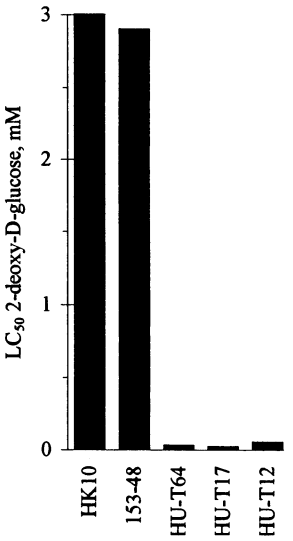

FIG. 6. 2-Deoxyglucose toxicity. Wild-type *Volvox* (HK10), the parent *NitA*$^-$ strain (153-48), and three HU-T transformants were grown in the presence of different 2-deoxyglucose concentrations. Concentrations at which 50% of the algae died are given (LC_{50}).

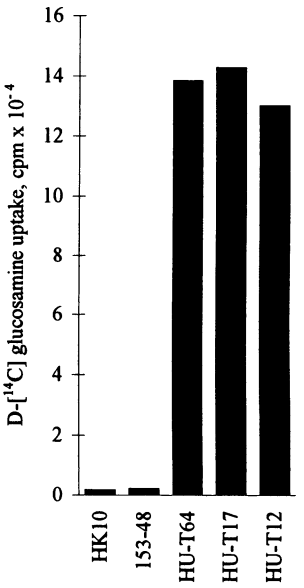

FIG. 7. [^{14}C]Glucosamine uptake assay. Wild-type *Volvox* (HK10), the initial *NitA*$^-$ strain (153-48), and three HU-Ts were grown in the presence of D-[U-^{14}C]glucosamine. D-[^{14}C]Glucosamine uptake is given in cpm per 10 *Volvox* spheroids per 60 min.

DISCUSSION

Labeling experiments using radioactive precursors like sugars are crucial to identify and study the developmentally controlled synthesis of unknown proteins, glycoproteins, lipids, and carbohydrates. Transgenic *Volvox* algae synthesizing a functional sugar transporter (HUP1) from *Chlorella* under the control of the constitutive *Volvox* β-tubulin promoter were able to import glucose, glucosamine, and 2-deoxyglucose, in contrast to their wild-type ancestors. These transgenic *Volvox* cells, to our knowledge the first that express a gene of heterologous origin, now allow the efficient application of [^{14}C]glucose in pulse- and pulse–chase-labeling experiments.

Earlier studies with the glucose transporter HUP1 were performed after its heterologous expression in the fission yeast *Schizosaccharomyces pombe* (25). A mutant strain of *S. pombe* was used for functional studies of the hexose/H^+ symporter (26, 27). Due to the absence of endogenous sugar transporters, transgenic *Volvox* offers an algal model system for mechanistic studies. The transgene should be equally useful for those studying metabolism in *Chlamydomonas*, which also is unable to use exogenous sugars.

The chimeric gene construct encoding the HUP1 hexose/H^+ symporter under the control of the *Volvox* β-tubulin promoter should also turn out to be a valuable tool for genetic experiments. Wild-type *Volvox* organisms die after prolonged incubation in the dark, whereas transformants survive provided glucose is added to the growth medium. This fact may allow the use of the glucose transporter as a selectable marker in transformation experiments not only for *Volvox* but possibly also for other photoautotrophic green algae such as *Chlamydomonas*. Additional selectable markers, besides nitrate reductase, are essential to establish insertional mutagenesis or gain-of-function experiments in *Volvox*.

In contrast to most other eukaryotes, *Volvox* exists as a haploid multicellular organism for most of its life cycle, a fact offering important advantages for genetic work. Up to now, however, the technique of gene targeting or gene "knockout" was not established in *Volvox*. In many higher eukaryotes, an artificially introduced DNA molecule carrying a mutant gene replaces with relatively low probability the corresponding wild-type gene by homologous recombination (28, 29). Usually gene addition instead of gene replacement occurs much more frequently for unknown reasons. Therefore, an efficient selection system is necessary to detect the few "knockout" organisms produced by the rare event of homologous recombination. Such a system normally employs two selectable markers, one that confers selection advantage, like the *Volvox* nitrate reductase gene and the other that confers selection disadvantage (30). This latter task can be done by the *Chlorella HUP1* gene, if cells are grown in the presence of 2-deoxyglucose. Thus all components are available now for gene targeting in *Volvox*.

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