GENERATION, CHARACTERIZATION AND RISK ASSESSMENT OF TRANSGENIC TILAPIA WITH ACCELERATED GROWTH

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ABSTRACT

Marine Biotechnology is a fast developing area in modern Biotechnology. Recent advances in gene transfer has offered the possibility to manipulate growth in fish through the transfer of chimeric growth hormone (GH) genes. We have generated several transgenic tilapia lines by the transfer of constructs containing the tilapia GH cDNA or chromosomal gene. A transgenic tilapia line with accelerated growth was obtained and characterized. The risks associated with the work in transgenic fish and shellfish were evaluated and measures were taken to conduct new experiments before introducing transgenic tilapia into the national aquaculture program.

Key words: transgenic, fish, tilapia, hormone

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RESUMEN

La biotecnología marina es un área de rápido desarrollo dentro de la Biotecnología moderna. Los avances recientes en las técnicas de transferencia de genes han posibilitado la manipulación del crecimiento en peces mediante la transferencia de genes de hormona de crecimiento (HC) quiméricos. Nosotros hemos generado varias líneas de tilapia transgénicas mediante la transferencia de construcciones que contienen el ADNc o el gen cromosomal que codifica para la HC de tilapia. Se obtuvo y caracterizó una línea de tilapia transgénica con mayor velocidad de crecimiento. Los riesgos asociados con el trabajo con peces transgénicos fueron evaluados y se tomaron medidas para realizar nuevos experimentos antes de introducir las tilapias transgénicas en los planes de desarrollo acuícola del país.

Palabras claves: transgénesis, peces, tilapia, hormona

Introduction

Recent Advances in Marine Biotechnology

The main topics of interest in the field of marine biotechnology could be focused in the following areas (1, 2):

a. Transgenic fish principles and applications.
b. Metamorphosis and reproductive activity in marine organisms.
c. Disease related problems in aquaculture.
d. Molecular Biology, genetics and cell culture.
e. Bioactive substances from marine organisms.
f. Utilization of marine photosynthetic organisms for food and feed production.
g. Marine based industrial materials.
h. Hydrothermal vent microbiology.
i. Bioluminescence.

Of these, the Center for Genetic Engineering and Biotechnology (CIGB) is involved in the areas a-d, although the most developed area is the first one.

a. Transgenic fish principles and applications

The generation of transgenic fish with chimeric growth hormone (GH) transgenes for manipulating growth performance in economically important species is the main goal of this area.

The present state of the art is mainly focused on:

- The search for appropriate transgene regulatory sequences.
- The use of "all fish" transgenes.
- The expression of transgenes introduced by microinjection or electroporation in fish embryos.
- The study of new methods for introducing foreign DNA into fish embryos (sperm cells as vectors).
- The inheritance and expression of heterologous genes.
- The use of GH genes in transgenic fish experiments.
- The construction of fish "bio-reactors".

b. Metamorphosis and reproductive activity in marine organisms

This field is focused on the characterization of signal molecules, chemosensory receptors and signal transducers controlling settlement and metamorphosis of the larvae of mollusks, corals, shrimps, lobsters and other organisms. It also studies the peptide pheromones and hormones that regulate reproductive activity in various marine species.

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Protein fractions in the digestive tract of the mollusk *Pecten maximus* and in the hepatopancreas of the lobster *Panulirus argus* have been found to immunoreact with an antisera directed against human insulin and hGH (3, 4). Furthermore, the immunoreactive protein fractions promote growth acceleration in the larvae of the shrimp *Penaeus schmitti* when supplied in the diet (3) and a recent publication reports on the effect of dietary rGHs on *Penaeus vannamei* and other shellfish larval development. These preliminary results, together with the identification of peptide GH in mollusks, allow for the cloning and utilization of these GHs and/or fish-derived GHs (5-10) and insulin-like genes for the production of recombinant proteins for diet supplementation or for the generation of transgenic organisms. Transgenic principles for these species are to be established. However, recently we reported the transient transformation of the shrimp *P. schmitti* (11) as a first step for the generation of transgenic shrimps.

c. Disease related problems in aquaculture

One of the major factors that will determine whether the aquaculture industry will succeed is disease control. To meet this need, many different biotechnological approaches are currently being applied in the control of diseases in fish and shellfish. They include the use of Mab and nucleic acid probes for improved diagnosis of disease and the cloning of important genes which may be subsequently expressed for subunit vaccine production or for the development of transgenic animals which are resistant to pathogens.

d. Molecular Biology, genetics and cell culture

All the projects listed above have experiments dealing with Molecular Biology, genetics and cell culture. We have tested the constructs employed for transgenesis in fish cell lines and embryos to determine the appropriate regulatory sequences for transgene expression (12-15). Genetics in economically important fish species is being addressed by many groups throughout the world. In tilapia, groups in Cuba have reported good results in response to selection (16).

Growth Hormone Gene Transfer In Fish

The era of effective gene transfer in animals began in 1980 with the pioneer work of J. Gordon (17). Since then, gene transfer technology has produced a great impact in modern molecular biology and biotechnology (18, 19). In the past few years, significant progress has been made in transgenic fish studies (20, 21). In particular, many efforts have been directed towards growth manipulation in fish because of the possible impact of these improved strains in aquaculture. Growth hormone gene transfer has been accomplished in several fish species (17, 21, 22). Recently, size increases in transgenic fish have been reported in some species (Table 1). However, because of the high variability in size and weight of fish (17), results from experiments assessing growth should be cautiously taken, unless evidence correlating the expression of the GH transgene and the phenotypic effect observed are supplied. Finally, the use of fish GH genes will probably give better results than mammalian genes and cDNAs.

Table 1. ***Results showing growth enhancement in fish in GH gene transfer experiments.***

Gene construct	Fish species	Size increase	Inheritance	Reference
mMT-hGH	loach, carp	3-4 fold	yes	23-25
RSV-tGH	carp	20 %	yes	26
		-2 %-59 %	yes	27
AFP-sGH	atlantic salmon	4-6 fold	ND	28
RSV-bGH	northen pike	yes	ND	29
chßactin-hGH	medaka	yes	yes	30
OnMT-GH1	coho salmon	11 fold	ND	31
CMV-tiGH	tilapia	80 %	yes	32, 33

Abbreviations: mMT, mouse metalothionein promoter; OnMT, sockeye salmon MT promoter; RSV, Rous sarcoma virus LTR; hGH, human GH; tGH, trout GH; AFP, antifreeze protein promoter; sGH, salmon GH; bGH, bovine GH; GH1, sockeye salmon type-1 GH gene; chßactin, chicken ß-actin promoter; CMV, cytomegalovirus; tiGH, tilapia GH.

Table 2. ***Gene transfer experiments in tilapia.***

Gene construct	DNA	Integration	Expression	Inheritance	Reference
mMT-hGH	Linear, 4 kb	yes	ND	ND	34
avianLTR-bGH	Linear, 3.5 and 8.5 kb	yes	ND	ND	36
mMT-rGH	Linear, 6.6 kb	yes	ND	ND	35
CMV-tiGH	Linear, 3.2 kb	yes	yes	yes	32
CMV±INT-tiGH	Linear, 6.8 and 6.2 kb	yes	yes	yes	32
RSV+INT-tiGH	Linear, 6.5 kb	yes	yes	yes	32
RSV-chrtiGH	Linear, 7.0 kb	ND	ND	ND	Martínez, *et al.* unpublished.

Abbreviations rGH, rat GH; INT, first intron from trout GH; chrtiGH, chromosomal tiGH gene; avianLTR, LTR from an avian retrovirus; ±, in the precense and absence of the intron. Other abbreviations, as in table 1.

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Gene Transfer in Tilapia Oreochromis sp.

Tilapia are economically important species accounting for over 70 % of the fresh water fish production in Cuba. Gene transfer in tilapia has been reported by different authors (32, 34-37) (Table 2). However, size increase in transgenic tilapia was not reported in these species until recently, when we presented our data at the International Marine Biotechnology Conference (1994) (32).

Generation of Transgenic Tilapia with tiGH Transgenes

The possibility of accelerating growth in tilapia was assayed by the exogenous administration of recombinant tiGH. The tiGH cDNA was cloned from hybrid tilapia pituitary glands and expressed in *E. coli* (38) and in the *Pichia pastoris* yeast (Lleonart *et al.* unpublished). The growth of juvenile tilapia (*Oreochromis sp.*) was accelerated (1.4 fold in length and 1.7 fold in body weight after 21 days) when the yeast-derived r-tiGH was administrated by three intraperitoneal injections at intervals of seven days. The control group received BSA injections.

For gene transfer experiments, chimeric constructs were prepared containing the tilapia growth hormone cDNA (tiGH) or chromosomal gene (chr-tiGH), 5' regulatory sequences derived from the human cytomegalovirus (CMV) or Rous sarcoma virus (RSV), polyadenylation sites from the SV40 and the first intron from the trout growth hormone gene (INT) (12, 13, 37). Employing these constructs, tiGH transient expression was obtained in mammalian and/or fish cells.

In previous studies we found that in *Xiphophorus* A2 cells CMV enhancer-promoter was more efficient than RSV sequences and that the presence of an intron is relevant for the RSV, but not for the CMV regulatory region (15). In contrast, in zebrafish embryos, the RSV regulatory region is 17 times stronger than the CMV, and again the presence of the intron is important only for the activity of the RSV enhancer-promoter (15). These results support the hypothesis that different regulatory requirements exist in cells and embryos and suggest that chimeric constructs designed for transgenic experiments should be assayed in transient expression experiments in fish embryos.

To evaluate the activity of different regulatory elements in transgenic fish, five lines of transgenic tilapia were generated (CMV>±INT>tiGH>SV40, CMV> tiGH> CAT>SV40 (CMV-tiGH), RSV> +INT>tiGH> SV40, and RSV>chr-tiGH) by direct microinjection of one cell embryos (32, Table 2). Although hormones generally work at low concentrations, the growth of the genotypes from the transgenic lines obtained with our different tiGH-containing chimeric constructs will have to be compared. Since we do not know the optimum constitutive GH levels required for better growth increase in tilapia, results will have to be obtained empirically comparing different constructs and transgenic lines in heterozygous and homozygous animals, characterizing the tissues and levels of ectopic tiGH expression.

Table 3. *Germ-line transmission of CMV-tiGH transgene in the transgenic "albino" tilapia line.*

Generation	Number of animals (transgenic / non-transgenic)	Inheritance
P1	1 (1 / 0)	--------
F1 (P1xWt)	38 (18 / 38)	47
F2 (F1xF1)	10 (8 / 10)	80
F3 ($F2^{+/-}xF2^{+/-}$)	10 (7 / 10)	70

Characterization of the "albino" Transgenic Tilapia Line

With the CMV>tiGH>CAT>SV40 construct (CMV-tiGH in Table 2), a transgenic animal containing 1 copy of the transgene per cell was selected to establish a transgenic line (F0-3 in reference 37 and later designated as "albino") (32, 33). The transgene was stably transmitted to F1, F2 and F3 generations in a Mendelian fashion (Table 3). Ectopic, low level expression of tiGH was detected in gonad and muscle cells of F1 transgenic tilapia by *in situ* hybridization and immunohystochemical analysis of tissue sections (33 and data not shown). Nine months old transgenic F1 progeny were 82 % larger than the non-transgenic ones at $p = 0.001$ (Table 4). Under different culture conditions, 4 and 7 months old F2 transgenic fish were 37 % and 55 % larger than non-transgenic siblings at $p = 0.01$ and $p = 0.009$, respectively. Transgenic F2 and non-transgenic siblings were compared with respect to feeding moti-

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Table 4. *Characterization of F1 progeny derived from transgenic "albino" tilapia.*

	Transgenic[a]	Non-transgenic[a]
N	18	20
% inheritance	47	--
Mean weight ± SE (g)	399.1±94.1	219.5±49.1
Weight range of the males (g)	219.2-710.0[b,]	95.9-472.1[d]
Larger than the largest control	44 % (8/18	--
Smaller than the smallest control	0	--
Mean growth increase[e] ($\delta_{average\ weight}/\delta T$)	50.4±4.2	28.5±2.5
% difference	81.8[f]	--

[a]Values for 9 months old tilapia in June 1994.
[b]75 % of transgenic males were larger than the largest control.
[c]N = 12, [d]N = 6.
[e]Mean±SD.
[f]Transgenic progeny were larger than non-transgenic progeny at p = 0.001.

vation (FM), dominance status (DS), 50 % of sea water adaptation (SWA) and digestibility coefficient (Guillén, *et al.* manuscript in preparation). Transgenic tilapia showed a higher FM ($p = 0.03$) and DS ($p = 0.0004$) than non-transgenic siblings. Transgenic F2 tilapia also showed a better adaptability to sea water, suggesting that GH could be involved in osmotic regulation in these species. No abnormalities have been recorded in this transgenic line. These results showed that low level ectopic expression of tiGH resulted in a growth acceleration in this transgenic tilapia line.

Ecological and Regulatory Issues associated with Transgenic Fish and Shellfish

Consideration of the Safety/Risks Relation of Working with Transgenic Fish and Shellfish

Although transgenic tilapia have been manipulated to accelerate growth, many characteristics of the parental organism should be maintained. We have not seen any abnormalities in transgenic tilapia, including analysis of homozygous animals. Food consumption, ecological impact and other aspects of these transgenic tilapia strains will have to be assayed under experimental conditions before introducing this technology into the national aquaculture programs.

The current knowledge does not allow us to predict the full range or magnitude of phenotypic changes in particular lines of transgenic fish. In the case of the transfer of GHs, impact pathways on the ecosystem could include the following (39): The large size of individuals bearing introduced GH genes might favor them in agonistic interactions over food resources. Because GHs directly and indirectly affect behavior, transfer of such genes could impact the successful execution of life history-related events such as migration, territoriality and mating.

Altered resources or substrate uses could result if, through accelerated growth, predatory transgenic individuals exhibit a larger mouth gap and make use of new prey items.

Therefore, there is a need for carefully evaluating the safety/risks relation in the research and introduction of transgenic fish and shellfish into national aquaculture programs.

A recent experiment comparing the DS of transgenic F2 and wild type (those directly obtained from natural ponds and not grown under laboratory conditions) tilapia suggested that transgenic populations accidentally escaping to natural ecosystems would have less chance to survive (Guillén, et al. manuscript in preparation). Wild type tilapia showed a higher DS than both transgenic and non-transgenic siblings ($p = 1.13$ E-12, ANOVA Single Factor Test). These preliminary observations addressed some of the concerns discussed (39) for the introduction of transgenic fish into the national aquaculture programs.

Performance Standards for Safely Conducting Research with Genetically Modified Fish and Shellfish

To conduct studies outside the laboratory, we held an *ad hoc* committee meeting devoted to the analysis of the conditions for releasing tilapia with accelerated growth in Cuba. This committee concluded that, under the conditions found in Cuba, little or no effect on the natural population will occur as a result of the accidental escape of transgenic fish, mainly because these natural populations do not exist and most of the fish species found now in the country have been introduced. Nevertheless, the committee recommended to follow the final draft of the "Performance Standards for Safely Conducting Research with Genetically Modified Fish and Shellfish" (documents No. 95-01 and 95-02, reference 40) prepared and released on April 15, 1995 by The U.S. Department of Agriculture, the Agricultural Biotechnology Research Advisory Committee, and the Working Group on Aquatic Biotechnology and Environmental Safety.

These standards, as they state in the overview, are accompanied by flowcharts that guide the assessment pathway in a way that allows to consider and implement the necessary measures to safely conduct the experiments with transgenic fish to accumulate the data needed to fully characterize these new fish strains. Our experiments have been planned and conducted following these recommendations (appendix 1).

The Importance of the Public Opinion

The public must be informed about the possibilities and limitations of transgenic technology. In Cuba, where the population is well educated, many people are aware of what genetic engineering and gene transfer mean. In the last 15 years, Cuba has experimented a rapid and successful development in modern biotechnology (41, 42). This effort together with widespread information on biotechnology and related technologies (43) have helped the public opinion to become favorable to these technologies and confide in their safety and results.

Public confidence in the results of performance tests and ecological risk analyses, in addition to the results themselves, will prove critical in determining whether transgenic fish and shellfish enter into the national aquaculture programs.

The scientific community has a great responsibility in the conduction and evaluation of experiments with transgenic organisms and in the education of the people to increase public confidence and to avoid extreme attitudes against the development and use of transgenic animals.

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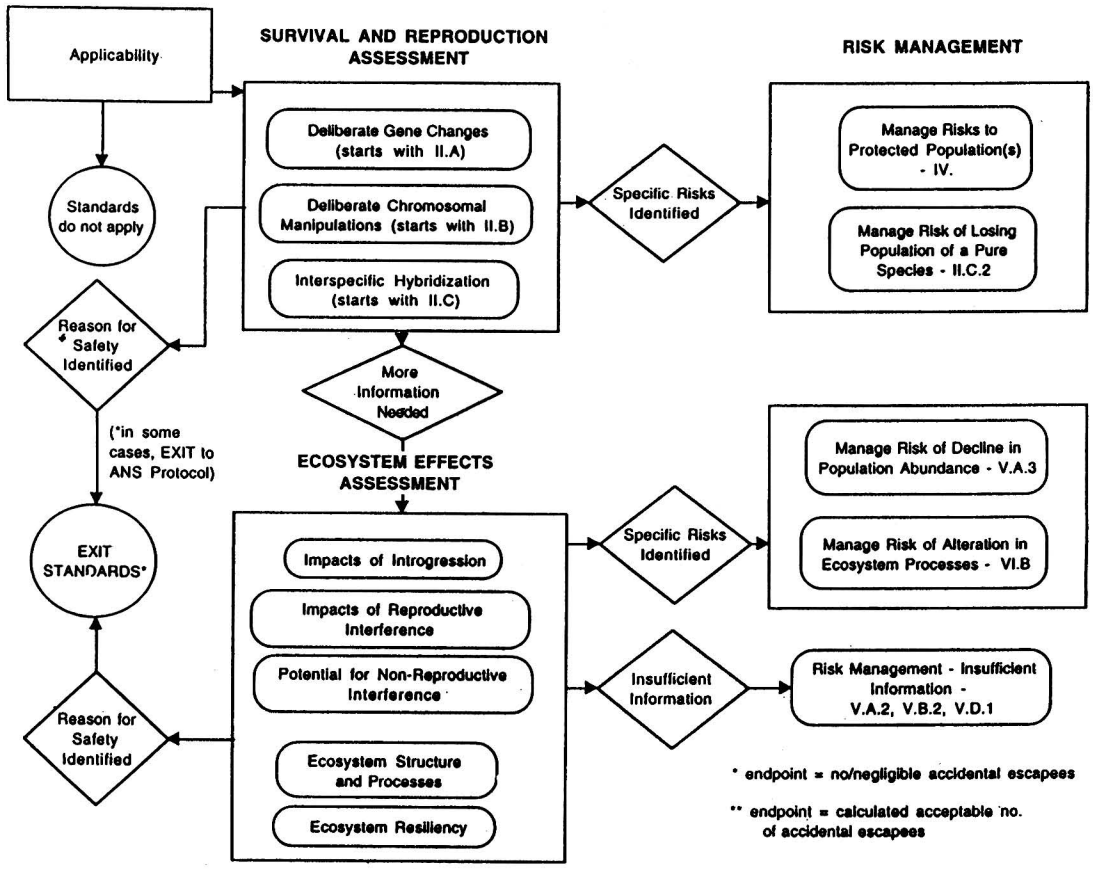

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Appendix

Worksheet Accompanying Performance Standards for Safely Conducting Research with Genetically Modified Finfish and Shellfish

Introduction

The Performance Standards for Safely Conducting Research with Genetically Modified Finfish and Shellfish are voluntary guidelines intended to aid researchers and institutions in assessing the genetic and ecological effects of research activities involving genetically modified fish, crustaceans, and molluscs, and in determining appropriate procedures and safeguards so that the research can be conducted without causing adverse impacts on the environment. The Flowcharts of the Performance Standards guide researchers in identifying, assessing and managing specific risks. This Worksheet accompanies the Flowcharts. Once completed by the researcher, the Worksheet will document both the decision path taken through the flowcharts of the Performance Standards, and any risk management measures. It is designed to assist researchers and reviewers in evaluating the project. Until the Performance Standards are incorporated into a computerized expert system with the capability of producing a hard-copy trace of the decision path, this worksheet should be used.
Principal researcher: José de la Fuente

Proposed Project
Field Testing of Transgenic Tilapia Expressing the Tilapia Growth Hormone cDNA in Cuba.

Please mark your response to a question by checking "Yes," "No," "Don't know," "EXIT," or by indicating your routing to a subsequent flowchart. The marking of more than one blank may be appropriate in particular situations. Attach written explanatory material as directed below.

Flowchart Documentation
Please list the number of all flowcharts that you used:
I., II.A., II.A.1., IV.A., IV.A.1., VI.B.

Flowchart
No.

I. Do the performance standards apply to the proposed experiment?
- __√__ Yes or don't know. Where were you routed?
  - __√__ Continue at flowchart II.A.
  - _____ Consult Appendix B.
- _____ No. EXIT the standards.

II.A. Does the GMO result from deliberate gene changes?
- __√__ Yes. Where does flowchart II.A. route you?
  - __√__ II. A.1. Assess impact of deliberate gene changes.
  - _____ EXIT the standards. *Attach your rationale.*
  - _____ No. Continue at flowchart II.B.

II.A.1. Where are you headed following completion of the flowchart regarding possible impact of deliberate gene changes? *Attach a written description of any identified risk.*
- _____ II. Assess potential interference with natural reproduction.
- __√__ IV.A. in Ecosystem effects assessment.
  Accidentally escaped GMOs may establish populations producing a potential for introgression.
- _____ IV.B. in Ecosystem effects assesment.
  Accidentally escaped GMOs may establish populations producing adverse effects on ecosystem structure or processes.
- _____ VI.A. Risk management-identified risks: Manage risks to protected.populations.
- _____ VI.B. Risk management-insufficient information.
- _____ EXIT the standards. Attach your rationale.
- _____ EXIT to Aquatic Nuisance Species Protocol (Appendix A)

II.B. Does the GMO result from deliberate chromosomal manipulations?
- _____ Yes. Where does flowchart II.B. route you?
  - _____ II.B.1. Assess potential impact of chromosomal manipulations.

Flowchart
No.

_____ II.C. Assess impact of additional modifications.
_____ EXIT the standards. *Attach your rationale.*
_____ No. Continue at flowchart II.C.

II.B.1. Where are you headed following completion of the flowchart regarding possible impacts of deliberate chromosomal changes? *Attach a written description of any identified risks.*
_____ III. Evaluate potential interference with natural reproduction.
_____ EXIT the standard. *Attach your rationale.*
_____ EXIT Aquatic Nuisance Species Protocol (see Appendix A).

II.C. Does the GMO result from interspecific hybridization?
_____ Yes. Where does flowchart II.C.route you?
_____ II.C.1. Assess potential impact of interspecific hybridization.
_____ EXIT the Standards. *Attach your rationale.*
_____ No. EXIT the standards. *Attach your rationale.*

II.C.1. Where are you headed following completion of the flowchart regarding potential impact of interspecific hybridization? *Attach a written description of any identified risks.*
_____ III. Evaluate potential interference with natural reproduction.
_____ VI.A. Risk management-specific risks: Manage risks to protected population.
_____ VI.A. Risk management-specific risks: Manage risks of losing population of pure species.
_____ EXIT the standards. *Attach your rationale.*
_____ EXIT to Aquatic Nuisance Species Protocol.

III. If you were directed to use the flowchart regarding potential interference of a sterile GMO with natural reproduction, where would you be routed? *Attach a written description of any identified risks.*
_____ IV.C. Ecosystem effects - impacts of reproductive interference.
_____ VI.A. Risk management-specific risks. Manage risks to protected populations.
_____ EXIT the standards. *Attach your rationale.*

IV.A. If you were directed to use the flowchart regarding potential ecosystem effects of GMOs expressing deliberate gene changes, where would you be routed?Attach material describing risks identified.
__√__ IV.A.1. Ecosystem effects-impacts of introgression of modified gene(s).
_____ VI.B. Risk management-insufficient information.
_____ EXIT the standards. *Attach your rationale.*

IV.A.1 If you were directed to use the flowchart regarding potential impacts of introgression of the modified gene into natural populations, where would you be routed? *Attach a written description of any identified risks.*
_____ V. Assess effects on ecosystem structure and processes.
_____ VI.A. Risk management-specific risks. Manage risk of decline in population abundance.
__√__ VI.B. Risk management- insufficient information.

IV.B. If you were directed to use the flowchart regarding potential barriers to reproduction of the GMO associated with the accessible ecosystem, where would you be routed? *Attach a written description of any identified risks.*
_____ IV.B.1. Ecosystem effects-potential for non-reproductive interaction.
_____ EXIT the standards. *Attach your rationale.*

IV.B.1. If you were directed to use the flowchart regarding the potential for non-reproductive interaction of the GMO with conspecifics or closely related species, where would you be routed? *Attach a written description of any identified risks.*
_____ V. Effect on ecosystem structure and processes.
_____ VI.B. Risk management - insufficient information.
_____ EXIT the standards. *Attach your rationale.*

IV.C. If you were directed to use the flowchart regarding potential ecosystem impacts of reproductive interference by sterile GMOs, where would you be routed? *Attach a written description of any identified risks.*
_____ VI.A. Risk management-specific risks. Manage risks of decline in population abundance.
_____ VI.B. Risk management-insufficient information.
_____ EXIT the standards. *Attach your rationale.*

Flowchart
No.

V. If you were directed to use the flowchart regarding potential effects of the GMO on ecosystem structure and processes, where would you be routed?

_____ VI.A. Risk management-specific risks. Manage risks to protected populations.
_____ VI.A. Risk management-specific risks. Manage risks of alteration of ecosystem processes.
_____ VI.B. Risk management-insufficient information.
_____ EXIT the standards. *Attach your rationale.*

VI.A. If you were directed to use the flowchart regarding risk management when there are identified risks, what measures do you plan to adopt to manage these potential risk(s)? *Attach a written description of the risk management measures you plan to implement. Be certain to address the topics listed in the Risk Management Documentation section below.*

VI.B. If you were directed to use the flowchart regarding risk management when there is insufficient information to assess risks, what measures do you plan to adopt to effectively confine the proposed experiment? *Attach a written description of the risk management measures you plan to implement. Be certain to address the topics listed in the Risk Management Documentation section below.*

Additional Questions

1. Are you working with a non-indigenous species?

_____ Yes.
_√_ No.

List names addresses, telephone numbers, and area of expertise of the persons you contacted for substantial advice in assessing effects of a proposed experiment and in designing adequate safety measures.
See the list of experts in acknowledgements.

Signature of researcher — June 2, 1995 (Date)

Address and Phone No. Division of Mammalian Cell Genetics. Centro de Ingeniería Genética y Biotecnología. P.O. Box 6162. Havana 6, CUBA
Phone No.: 21 6221
Fax No.: 218070
E-mail: invest@serverdos.cigb.edu.cu

Risk Management Documentation

As part of the compliance with the voluntary Performance Standards, the researcher must describe and provide the rationale for the risk management measures. Major points explained in the text on Risk Management Recommendations are listed below. Researchers and reviewers should read the text on Risk Management Recommendations before using this portion of the Worksheet. The risk management documentation must fully respond to these major points. For items which request a narrative response, attach your written responses and identify the numbered item being addressed.

Project Setting

1. Explain how the setting and structures of the project prevent accidental releases during flooding or other natural disasters.

A. If project involves placement of GMOs in uncovered outside tanks or ponds, is there the potential for sudden high winds to wash organisms into a natural water body (accessible ecosystem) via water spray or waves?

_____ Yes. Proceed to item 1.b.
_√_ No. Proceed to item 2.

B. If there is potential for GMOs held in outside units to be washed via sudden high winds into a natural water body, what measures will be taken to adequately cover these outside units or otherwise protect against movement of GMOs by water spray or waves into nearby natural water bodies? (Explanatory diagrams may be useful).

Design of Barriers

The Standards identify four types of barriers: (1) physical or chemical; (2) mechanical; (3) biological and (4) scale of experiment as a barrier.

2. Was the project site chosen because the surrounding accessible ecosystems are lethal to all life stages of the GMO?

_____ Yes. Address items 2.a and 2.b.
√ No. Proceed to item 3.
(a) Describe evidence that the accessible ecosystems are indeed lethal to the GMO.
(b) Explain how the setting reduces the need for barriers on-site.

3. Could the project's GMOs potentially escape through any of the paths (aquatic and non-aquatic) listed below? Answer "Yes" if there is potential for escape or uncertainty about potential escape of GMOs via the listed path. Answer "No" only if escape is clearly precluded.

Yes a. Influent/makeup water?
Yes b. Effluent and drawdown water?
(Note: if discharge to sanitary sewer is used as one barrier against accidental escape of GMOs in effluent, at least one additional barrier is necessary)
No c. Waste slurries?
No d. Disposal of experimental animals?
No e. Aerosols (applies only to shellfish with small larvae)?
No f. Equipment cleaning and storage?

4. Have you identified additional, potential escape paths? If so, briefly describe each path.

5. For each escape path identified in items 3 and 4 above, describe the arrangement and types of barriers to escape; a diagram of layout of barriers at the site or facility may be useful. Describe: treatment and disposal of waste slurries; disposal of experimental animals; and cleaning and storage of equipment.

6. Describe how the types and numbers of barriers in series are sufficient to achieve the "acceptable number of accidental escapees" specified in Flowcharts VI.A. or VI.B.

Special Concerns

7. Yes If biological barriers are used for a given escape path, does the path have at least one other type of barrier? (Because of their variable efficacy, biological barriers cannot comprise the entire set of barriers).

8. n.a. If scale is used as a barrier, are you certain the GMO is not a self-fertilizing hemaphrodite or true parthenogen? Attach supporting evidence.

Security

9. Describe the security measure implemented to:
a. Control normal movement of authorized personnel,
b. Prevent unauthorized access to the site, and
c. Eliminate access for predators who could potentially carry animals off-site (applies only to outdoor projects).

Alarms

10. Describe and justify the adequacy of the entire set of installed alarms. Be sure to address the following:
a. Have you installed a water level alarm (required for all projects)?
b. Do all installed alarms have backup power?
c. Describe the plan for notifying designated personnel.

Operational Plan

11. Attach the written operational plan. Required components are:
a. Training.
b. Traffic Control.
c. Record Keeping.
d. Emergency Response Plan.

Review and Inspection

12. Has your institutional biosafety committee, biosafety officer, or other appropriate expert reviewed and approved the proposed project and its risk management measures? If not, explain the review status of your project.

√ Yes
___ No

Have you notified federal, state, and local agencies having jurisdiction over any aspects of your proposed project? If not, please explain.

√ Yes
___ No

Please list all required permits and authorizations and check appropriate line regarding status of your application:

√ approved
___ pending
___ not yet submitted

Summary of the Supporting Material Attached to

Worksheet Accompanying Performance Standards for Safely Conducting Research with Genetically Modified Finfish and Shellfish.

Proposed Project
Field testing of transgenic tilapia expressing the tilapia growth hormone cDNA in Cuba.

Summary of the Risks Identified and Proposed Measures

Risks identified: The accessible ecosystems contain co-specific species with which transgenic tilapia could breed. Since transgenic aninals are fertile, there is a risk for a potential introgression of the chimeric tilapia growth hormone gene into natural populations. Nevertheless, in the case of Cuba, tilapia were introduced in the sixties and there is no risk of affecting endogenous species. We are dealing with ecosystems deliberately modified.

However, because we do not have all the information, we decided to evaluate the reproductive potential, genetic flow and behavior of transgenic tilapia while preventing the accidental escape of these genetically modified organisms.

Documentation for risk management: The proyect will be conducted in artificial ponds in G and M in the province La Habana. Both sites guarantee the safety required for the experiment (eg. location above the 100 year flood level).

G: This site possesses artificial ponds of 6 and 140 m^2. Effluents and drawdown water discharge directly to the ocean through a 4 Km canal. No other surface water bodies are located in the area. Mechanical, chemical and biological barriers were placed at the initial and final points of the water system of the project. This site will be used for the conformation of the reproduction stock and for the evaluation of the industrial process $T^{+/+}$ x wt.

M: This site possesses artificial ponds of 0.4, 0.8 and 1 ha. The water comes through gravity from a large artificial water body and the effluents and drawdown water discharge directly to the ocean through a 5 Km canal. Mechanical barriers were placed at the initial and final points of the water system of the project. The 0.4 ha pond will be used in a second part of the project for the evaluation of transgenic heterozygous tilapia ($T^{+/+}$ x wt) under extensive culture conditions. Intensive culture conditions will be evaluated under controlled laboratory conditions.

In both sites the authorized personnel involved in the project will be controlled to prevent unauthorized access to the site. Security guards and other security measures will be also implemented.

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