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Gene Transfer and Cauliflower Mosaic Virus Promoter 35S Activity in Mammalian Cells

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The cauliflower mosaic virus 35S promoter (CaMV35s) is extensively used in genetically modified crops for human and animal consumption. Horizontal gene transfer is attracting particular attention, in light of experimental reports, showing the presence of dietary DNA into animal tissues. Health implications may derive from possible activities of the heterologous promoter in mammalian cells after integration in the host genome. To evaluate this hypothesis, in vivo and in vitro experiments were performed using GFP as reporter gene. Recombinant plasmid DNA was fed to Balb/c mice and searched in several tissues by PCR amplification. The activity of the plant virus promoter was assessed by RT-PCR and fluorescence microscopy after liposome-mediated transfection of murine gonadic cells. Obtained data did not highlight evidences of dietary DNA transfer in mice. No CaMV35s transcriptional activity was detected in this experimental model. These findings emphasize the need for further studies and standardized methods.

Key Words: Cauliflower mosaic virus 35S promoter; Gene transfer; Genetically modified organisms; Public health; Biotechnology; Novel food.

INTRODUCTION

Transgenic plants are a fact of modern agriculture and paradigmatic examples of biotechnological achievements deeply influencing our modern societies. Scientific breakthroughs often come together with passionate debates. Current public consideration for genetically modified (GM) organisms ranges from optimistic expectations to worrisome fears.[1]

A major issue associated with the production and consumption of transgenic crops is related to gene transfer and its possible consequences for human

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health and environment.[2,3] The present report takes place in a puzzling picture of contrasting published data.[4,5] With no claim to exhaustiveness, the experimental approach focuses on both gene transfer from food to animal tissues and evaluation of transcriptional activity of the cauliflower mosaic virus 35S (CaMV35S) promoter in mammalian cells. The CaMV35S promoter was originally cloned from a plant virus and is extensively used in genetically modified crops for human and animal consumption.

Several papers have pointed out how DNA fragments of dietary origin can survive degradation within the digestive tract of animals and humans.[6,7] Heterologous DNA sequences, for instance, were detected by molecular techniques in blood, feces, milk, and/or various organs of mice, poultry, and cattle.[8,9] Similarly, in vitro digestion models have demonstrated the capability of DNA fragments to tolerate a simulated gut-mediated degradation.[10,11]

Thus, it is generally accepted that DNA degradation, along the gastrointestinal (GI) tract of animals, is not complete. Uptake and integration of these surviving nucleic acid fragments by blood cells and animal tissues cannot be ruled out, even under physiological conditions.[12,13] However, transcription of dietary fragments and in vivo uptake of transgenic elements are still debated and under investigation.[14] This study aims to evaluate the possible occurrence of dietary transmission of transgenic elements in mice and the in vitro activity of a GM plant promoter in mammalian cells.

MATERIALS AND METHODS

Cell Cultures

Cell lines used for the experiment were obtained from ATCC (USA). TM3 (CRL-1714) and TM4 (CRL-1715) are murine Leydig and Sertoli cells, respectively.[15] Propagation was performed in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium with 1.2 g/L sodium bicarbonate and 15 mM HEPES, 92.5%; horse serum, 5%; fetal bovine serum, 2.5%.

Transfections

TM3 and TM4 cell lines were grown and propagated, in the required medium, for several days prior transfection. Transfection was performed using DOTAP Liposomal Transfection Reagent (Roche, Germany) and following the manufacturer's general recommendations. The day before transfection, cells were subcultured, counted in a standard hemocytometer, and seeded in triplicate in a 24 well plate at a density of 10^6 cells/10 cm^2. Several preliminary experiments were conducted to identify the best transfection conditions suitable for the employed cell lines. As a result, an estimated ratio of 2.5 μg DNA/10^6 cells was finally considered. DOTAP reagent was used at a ratio of either 5 μl/μg

DNA or 10 μl/μg DNA. Cells were incubated with the DOTAP/nucleic acid mixture in standard culture medium for six hours. Successively, wells were gently but thoroughly washed twice with fresh culture medium. Fresh medium was then added to the wells. Cells were monitored 24, 48, and 72 h post-transfection, and the medium was replaced concomitantly. The transfection efficiency was estimated by manually counting the cells in the wells or in a hemocytometer. For molecular analyses, an aliquot of the medium (250 μl/well) was collected from the wells 24 h post-transfection when fluorescence was already observed. DNA was extracted from this sample by Proteinase K digestion followed by organic extraction.[16]

Plasmid Vectors

Plasmid pEGFP-C1 (U55763) is a 4.7 kb long commercial vector (BD Biosciences, USA), carrying a recombinant variant of the green fluorescent protein gene (EGFP) of *Aequorea victoria*, under control of the human cytomegalovirus (HumCMV) immediate early promoter. The plasmid was used as a positive control in transfections of mammalian cells with pBI121-GFP. This latter plasmid carries the EGFP gene, linked to the CaMV35S promoter, and was obtained within an interfaculty project of the Catholic University Medical School by subcloning the EGFP gene into a pBI121 vector (Clontech Laboratories Inc., USA) (AF485783) from where the gusA gene, encoding the β-glucuronidase, had been previously excised. The construct pBI121-GFP was checked by sequencing, and the expression of the reporter gene positively observed in plant cells (data not shown). DNA plasmid preparations were obtained using the DNA Maxiprep Kit (Promega, USA).

In Vivo DNA Uptake

In vivo experiments were conducted at the animal facility of the Catholic University Medical School of Rome upon approval of the experimental protocols by the Ethical Committee. Mice dietary regimen was constantly kept under strict observation, so that only standard irradiated food pellet and controlled water were fed to animals. Water was freely available throughout the study. During the experiment, litters were cleaned on a daily basis and consisted of standard sawdust. A total number of 40 animals were employed for the experimental protocol. Of them, 10 were used as controls. The day before the experiment at Time -12 (T_{-12}), healthy male Balb/C mice were selected, weighed, and starved overnight. All animals were three to four weeks old and weighed between 20 and 25 grams. The following day (T_0), animals were separated in individual cages and fed 50 μg of purified pEGFP-C1 (1 μg/μl). DNA was pipette spotted on a clean chip of standard irradiated feed pellet (about 2.5 grams/mouse). All animals received the same amount of DNA, except

Table 1: DNA uptake in vivo experimental protocol.*

Group	Number of animals	Diet	Experimental schedule	Harvested specimens
Treated	30	Irradiated feed +50 μg DNA	T_{-12}: starvation T_0: feeding T_{24}: feeding T_{48}: feeding T_{51}: sacrifice	Blood, liver, spleen, lungs, brain, intestine
Control	10	Irradiated feed +50 μl sterile TE buffer		

*40 mice were starved overnight and fed 50 μg of sterile DNA for three consecutive days (T_0 to T_{48}), apart from the control group that received 50 μl of sterile TE buffer. At T_{51}, that is, 3 h after the third meal from the beginning of the experiment, animals were sacrificed and the indicated biological specimens were collected for successive molecular analyses. Subscripts from 0 to 51 represent the hours elapsed after the first meal.

controls that were mock treated, in parallel, with 50 μl of sterile TE buffer. Animals were monitored for a few hours until they all finished their food supplies. Mice under study were fed the same way also 24 and 48 h later (T_{24} and T_{48}). About 3 hours after the third meal since the beginning of the experiment (at T_{48}), all the animals had finished their food (T_{51}) (Table 1). Mice were then sacrificed, and blood was collected promptly in EDTA for immediate DNA extraction. Organs were harvested using the maximum caution, in order to avoid DNA trans-contamination between specimens belonging to the same animal or to different ones. Either sterile disposable or autoclaved surgical instruments were used. For each animal, the following biological specimens were collected: blood, liver, spleen, lungs, brain, and intestine. Organs were snap frozen in liquid nitrogen to inhibit nucleases. Samples of the sawdust from litter and water were collected for molecular analyses. DNA was extracted from organs, litter, and water samples by Proteinase K digestion, followed by organic solvents extraction.[16] Prior analysis, DNA extracts were quantified spectrophotometrically, checked by agarose gel electrophoresis for purity and quality, and normalized by dilution in sterile, DNase-free water.

PCR

Primer pairs were designed using Primer Express 2.0 Oligonucleotide design software (Applied Biosystems, CA, USA): GFP-F1: TCC GCG TTA CAT AAC TTA CGG; GFP-R1: GAA CTC CAG GAC CAT GT; GFP-F2: GAG CAA GGG CGA GGA GCT GTT; GFP-R2: GTA CAG CTC GTC CAT GCC GAG AG; Beta-Actin Forward Primer (Mouse): TGT TTG AGA CCT TCA ACA CC; Beta-Actin Reverse Primer (Mouse): TAG GAG CCA GAG CAG TAA TC (murine β-Actin accession number: NM_007393). ETS1 primer pair was used as previously described.[17] Real Time PCR was performed by the Applied Biosystems Sequence Detection System 7000. SYBR Green PCR Master Mix and disposables were obtained from Applied Biosystems (CA, USA). For PCR assays, amplification reaction efficiency and absence of possible polymerase inhibitors were tested in parallel each time, adding serial dilutions of internal positive controls

(IPCs) directly to the samples. Negative and positive controls were always performed in parallel and consisted of sterile water or serial dilutions of purified pEGFP-C1 (2 ng to 1 fg), respectively.

cDNA Synthesis and RT-PCR

RNA was extracted from transfected cell monolayers, 72 h post-transfection, by the SV Total RNA Isolation System (Promega, USA) or TRIZOL Reagent (Life Technologies, USA), according to the protocol provided by the manufacturer. RNA quality and quantity were checked by agarose gel electrophoresis and spectrophotometric reading. cDNA was synthesized from normalized total RNA, using hexanucleotidic random primers and Superscript II RNase H Reverse Transcriptase (Invitrogen, USA).

RESULTS

CaMV35S Promoter Activity in Mammalian Cells—Fluorescence Microscopy

In transfected TM3 and TM4 cells, no evidence of CaMV35S promoter activity was observed by fluorescence microscopy. During several independent experiments, fluorescence in the transfected wells was monitored as a sign of expression of the green fluorescent protein, taken as a reporter. Several independent experiments gave reproducible results (Table 2). Fluorescence was detected only in positive control wells transfected with pEGFP-C1, carrying the HumCMV viral promoter. Although at the beginning, two different ratios of μl DOTAP liposomal transfection reagent/μg DNA were employed, a 5:1 ratio was successively chosen instead of 10:1. The lower ratio seemed to give better results, in terms of transfection efficiency, in all the optimization experiments. Wells were monitored until up to 72 h post-transfection, although the increase was small in terms of number of fluorescent cells attained after 48 h. TM3 and TM4 cell lines showed similar transfection efficiencies, and no significant differences were registered between the two cell lines employed.

CaMV35S Promoter Activity in Mammalian Cells—Molecular Methods

Molecular methods were employed to further investigate the lack of activity in mammalian cells of the viral promoter CaMV35S and corroborate the microscopic findings. DNA was extracted from all the transfected wells and tested by PCR for the presence of the plasmid vector pEGFP-C1, with vector-specific primers. Primers GFP-F1 and GFP-R1 were designed to specifically detect the pEGFP-C1 plasmid. The amplification protocol confirmed the presence of the pEGFP-C1 plasmid only in the wells transfected with such a vector.

Table 2: In vitro activity of CaMV35S promoter.*

	Mean observed fluorescence ($n = 3$) (% positive cells/well)					
	TM3 cell line			TM4 cell line		
	pEGFP-C1	pBI121-GFP	Mock transfection	pEGFP-C1	pBI121-GFP	Mock transfection
24 h post-transfection						
μl DOTAP/μg DNA Ratio = 5	30	0	0	40	0	0
μl DOTAP/μg DNA Ratio = 10	15	0	0	15	0	0
48 h post transfection						
μl DOTAP/μg DNA Ratio = 5	60	0	0	70	0	0
μl DOTAP/μg DNA Ratio = 10	35	0	0	30	0	0
72 h post-transfection						
μl DOTAP/μg DNA Ratio = 5	60	0	0	75	0	0
μl DOTAP/μg DNA Ratio = 10	38	0	0	30	0	0

*The mean percentage of fluorescence observed in three wells ($n = 3$), at 24, 48, and 72 h post-transfection is indicated. TM3 and TM4 cells were transfected in triplicate with two plasmid vectors as described in materials and methods. The table shows the results of a single experiment with two different DOTAP/DNA concentration ratios. A 24 well plate was used. Several further assays were performed, and consistent results were always obtained.

None of the DNA extracted from the wells incubated with pBI121-GFP gave a positive signal. These results also excluded the lack of unintentional contamination between wells during the experiment, as well as the lack of sequences homologous to pEGFP-C1 in both non-transfected (i.e., mock transfected) TM3 and TM4 cell lines. To verify the presence of the EGFP reporter gene in all the transfected wells, primer pair GFP-F2 and GFP-R2 was considered. Oligonucleotide primers were designed to anneal with a common fragment internal to the EGFP gene, carried by both pEGFP-C1 and pBI121-GFP. As expected, amplification of the DNA extracted from the wells showed the presence of the 700-bp-long EGFP-specific amplicon, in the cells transfected with both pEGFP-C1 and pBI121-GFP. DNA extracted from mock treated cells produced no amplification products.

The culture medium was tested to exclude the presence of the vectors used in the experiment. Aliquots of medium were collected from the transfection wells, 24 h post-transfection (i.e., when fluorescence was observed) and subjected to Proteinase K digestion and DNA organic extraction.[16] Purification products were then amplified with primers GFP-F2/GFP-R2. All the cultural media gave consistently negative results that endorsed the association of the analyzed DNA with the transfected cells harvested from the wells.

To study the expression of the reporter gene by molecular methods, a RT-PCR was carried out. RNA was extracted from the wells 72 h post-transfection, and cDNA was synthesized from normalized total RNA using hexanucleotidic

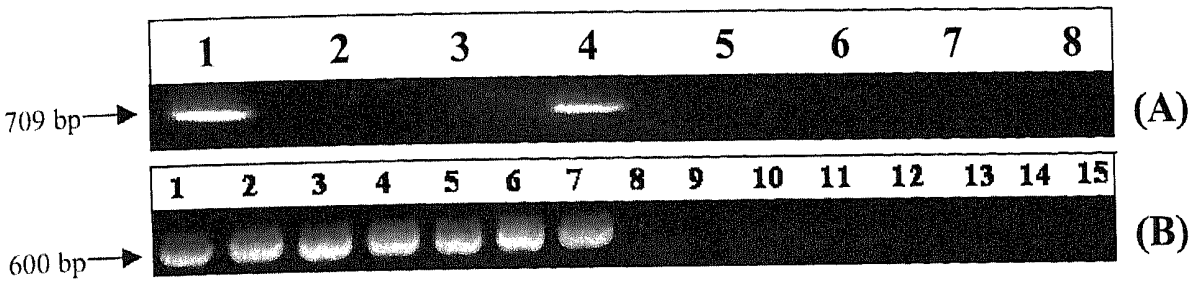

Figure 1: (A) EGFP specific RT-PCR from the transfection plates. cDNA was synthesized from the transfection plates and amplified using primer pair GFP-F2/GFP-R2, specific for the EGFP reporter gene. 1) TM3/pEGFP-C1; 2) TM3/pBI121-GFP; 3) TM3/mock transfected; 4) TM4/pEGFP-C1; 5) TM4/pBI121-GFP; 6) TM4/mock transfected; 7) Transfection water neg ctrl; 8) PCR neg ctrl. (B) β-Actin-specific RT-PCR from the transfection plates. cDNA was synthesized from the transfection plates and amplified using primer pair mouse β-Actin forward/mouse β-Actin reverse. RNA preps were tested for the presence of contaminating DNA by PCR amplification with the same primer pair (lanes 8–13). 1) TM3/pEGFP-C1; 2) TM3/pBI121-GFP; 3) TM3 mock transfected; 4) TM4/pEGFP-C1; 5) TM4/pBI121-GFP; 6) TM4 mock transfected; 7) β-Actin Pos Ctrl; 8) RNA Prep TM3/pEGFP-C1; 9) RNA Prep TM3/pBI121-GFP; 10) RNA Prep TM3 mock transfected; 11) RNA Prep TM4/pEGFP-C1; 12) RNA Prep TM4/pBI121-GFP; 13) RNA Prep TM4 mock transfected; 14) Transfection water Neg Ctrl; 15) PCR Neg Ctrl.

random primers. One EGFP-specific primer pair (GFP-F2/GFP-R2) and an additional primer set, specific for the β-Actin, were used to carry over the amplification reaction. Mouse β-Actin gene was chosen as the endogenous expression control. A second PCR was performed using a further primer set, specific for ETS1, an oncogene expressed in testes.[18] RT-PCR unambiguously confirmed the expression of the EGFP reporter gene, only in the wells where fluorescence was previously observed by microscopic observation. These wells were, again, those transfected with pEGFP-C1 plasmid, carrying the EGFP gene under control of the HumCMV promoter. No expression of the EGFP gene was observed when cDNA, from pBI121-GFP-transfected cells or mock transfected cells, was amplified with EGFP-specific primers (Fig. 1A).

Conversely, all the transfection wells showed similarly high levels of expression for the housekeeping gene β-Actin (Fig. 1B) and ETS1 gene. These results (data not shown) were also confirmed by Real Time PCR.

In Vivo Studies of DNA Uptake

Plasmid vector pEGFP-C1 lacks homology to mouse DNA and has already been used in previous studies focusing on the fate of dietary DNA, as a paradigmatic foreign DNA molecule. pEGFP-C1 plasmid was fed to mice. Attempts to localize specific fragments of the vector, in various mouse organs and blood, were performed by sensitive molecular techniques. Both traditional PCR and Real Time PCR, with SYBR green II and primers specific for EGFP gene (GFP-F2/GFP-R2), always failed to detect foreign DNA of dietary origin in all the tissues considered, namely, brain, spleen, lungs, liver, intestine, and blood. Irradiated feedstuffs fed to all animals, clean sawdust used for the litters and fresh water, sampled for control analyses, lacked DNA sequences homologous to

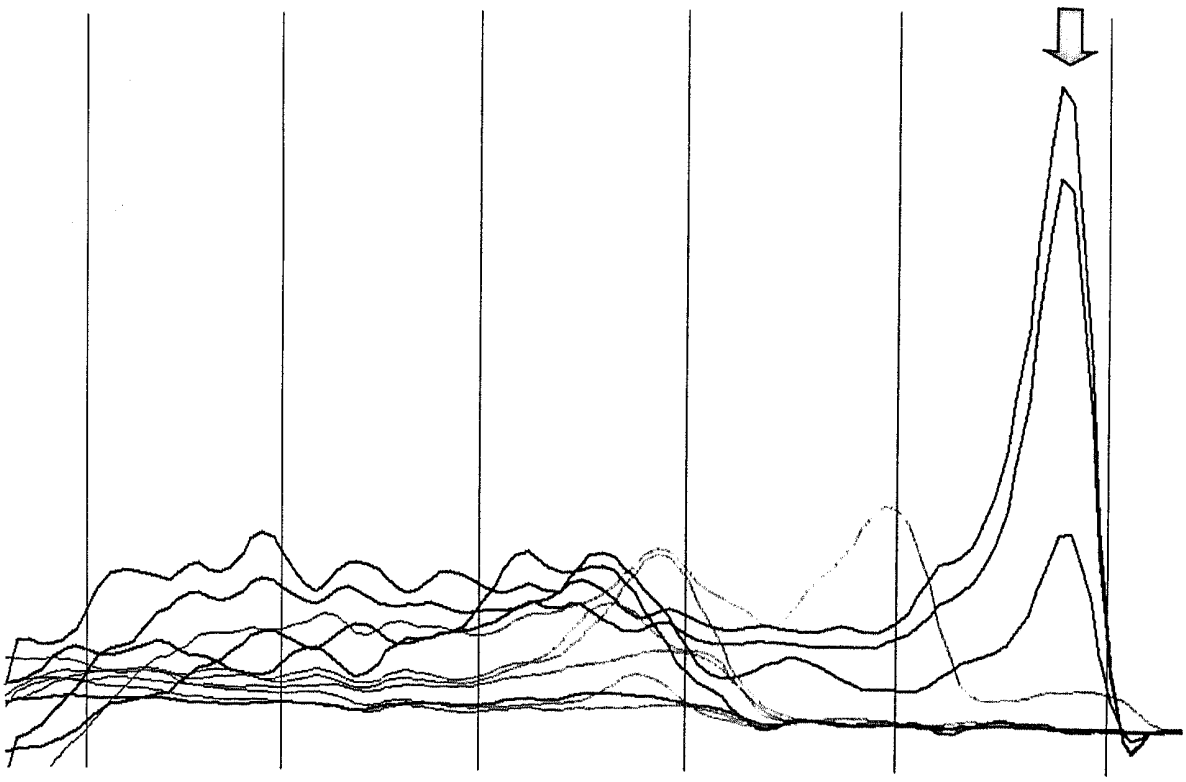

Figure 2: Real Time PCR of DNA, extracted from mouse specimens and control samples. Dissociation curves relative to DNA extracted from several biological specimens and control samples are shown. The curves show the measured melting temperatures of all the amplified sequences. Mice were fed purified pEGFP-C1 plasmid vector carrying the EGFP reporter gene. Detection of EGFP-homologous fragments in the indicated organs and specimens was performed with EGFP-specific primers. Serial dilutions of purified plasmid preparations were used as controls. As shown, based on the comparison of amplicon melting temperatures, EGFP-specific dissociation peaks were obtained for positive controls only.

EGFP (Fig. 2). Variable but constantly, slightly lower reaction efficiencies were observed for IPCs with respect to analogous dilutions of the positive controls. This was likely due to the presence of polymerase inhibitors that co-purified with the DNA from the biological specimens. The detection limit of the system, estimated by the IPCs, proved to be affected by the heterogeneity of the DNA preparations from organs. However, 10 fg to 1 pg of purified pEGFP-C1, intentionally added to DNA samples, were always detected.

DISCUSSION

Transfer and expression of transgenic elements from food to animal tissues appears unlikely to some authors.[11,12] Scientific evidences seem to demonstrate the existence of a tight biological barrier acting as a defense mechanism against foreign DNA uptake by germ cells in mammals.[13] Nevertheless, for the novelty of the problem, and the large number of people already exposed to edible transgenic crop, addressing this issue is generally considered critical.

This paper focuses on two different but related aspects associated with the consumption of foods obtained from transgenic plants: the possibility of

DNA transfer from food to animal tissues and the activity in mammalian cells of the CaMV35S viral promoter widely adopted in agrobiotechnology. Both the issues are linked together by the chain of events that would possibly lead to the expression of genes under the control of the ingested CaMV35S viral promoter.[12] In light of the current experimental findings, it seems questionable whether very low or no transcription activity of the CaMV35S promoter in mammalian cells can pose a significant threat for humans feeding on transgenic plants.[19,20] However, CaMV35S promoter, cloned from a plant virus, is present in practically all transgenic plants already commercialized and is reckoned by some authors as a hazardous genetic element.[21] CaMV35S functions efficiently in *E. coli*, green algae, and yeast, and despite its widespread employment in biotechnology, only a few studies so far have addressed the activity of the CaMV35s promoter in mammalian cells.[22]

We observed no evidences of activity of the CaMV35S promoter in murine gonadic cells by in vitro transfection experiments. A recent paper showed an extremely low but measurable in vitro activity in human 293T and, to a minor extent, 293 cells.[22] A further paper[20] reported relatively high levels of activity observed for the CaMV35s promoter in CHO cells with respect to the eF1α promoter. To account for this inconsistency, it should be considered that, between the experimental layout presented here and in the two other papers, several differences can likely constitute significant experimental variables. Gene expression assays, plasmid vectors, reporter genes, regulatory elements, and cell lines do not match exactly among the three studies. Moreover, stability of mRNA and consequent overall transfection efficiency are proven to be associated with proper termination codons.[23] The sequence carried by pBI121-GFP and used to halt the transcription of the EGFP reporter gene is the nopaline synthase terminator of *Agrobacterium tumefaciens*. This terminator is different from that used by Tepfer and co-workers.[20]

The possibility for the DNA to move from food to animal tissues, is supported by several intriguing reports on horizontal gene transfer, mainly by Doerfler and colleagues, and by the extreme conditions required for plant DNA degradation during food processing.[8,13,24] Taken together, these studies may have significant implications for public health and scientific knowledge.[7] In contrast to some previous publications, no evidences of DNA transfer from food to animal tissues were observed in our study. However, horizontal gene transfer to gut microflora or animal cells is a debated issue,[4] possibly lacking a commonly accepted monitoring approach.[25]

It is reasonable to speculate that experimental discrepancies are due to the difficulties associated with the design of a universally suitable protocol. In this respect, previous investigations have also pointed out the problems associated with the adoption of reliable approaches representing consistent and sensitive methods for forensic examinations or basic research.[9] For instance, previous

studies failed to obtain consistent results in all the animals or all tissues (within the same cohort), even when gene transfer was actually observed.[6,26] Thus, the experimental design should ultimately take into account both an appropriate model for the phenomenon and effective appraisal strategies.

Anatomical and physiological features of the GI apparatuses of various animal species can process dietary nucleic acids differently.[9] Food processing, ensiling, and/or storage time also show remarkable consequences on DNA stability. Thus, in vivo research can provide contradictory results if different animal models are employed in feeding experiments of feedstuffs that have been stored or processed differently.[27] Feeding schedules and food regimens are further misleading parameters in protocols, since food, in terms of both quality and quantity, can deeply affect the occurrence of gene transfer by extending the digestion time or by preventing DNA from being degraded. The inclusion of DNA in the food bolus or its possible association with food-derived proteins seems to protect it from enzymatic activity, and naked DNA has also been proven more sensitive to degradation by nucleases.[6] Although the amount of foreign DNA/mouse was the same as that used in other papers, mice employed in this study have been given only a limited amount of food throughout the time course (about 2.5 grams feed pellet/mouse/day). It cannot be ruled out that this dietary regimen may have somehow contributed to a consistent and rapid degradation of the DNA in treated animals. In addition, length of treatment, time of sacrifice, and tested organs can represent significant sources of data discrepancy.

The effectiveness of the strategies employed for gene transfer assessment can be significantly influenced by several factors, such as the target sequence of choice. Some studies have reported marked differences in target detection, produced by different primer pairs, for the test of the same sample. Chowdhury et al. have shown that, in calves fed GM maize, although inconsistently, two maize-intrinsic DNA fragments were detected in peripheral blood mononuclear cells, visceral organs, and longissimus muscle, while the transgene cry1Ab was never detected in PBMC or in visceral organs.[28] By contrast, a previous animal model failed to detect by PCR, both corn-intrinsic and recombinant DNA fragments, in the peripheral blood of pigs fed GM corn.[29] The choice of various amplicon lengths, number of primer pairs, and DNA extraction procedures can also be sources of conflicting data among the various studies.

Future research may shed light on possible influences of different drugs, gene delivery systems, or specific nucleotide sequences on gene transfer.[30,31] In light of current traceability regulations, it would be desirable to develop novel biomarkers and sensitive molecular techniques to monitor foods and the food chain for the possible presence of transgenic elements introduced with fodder, birdseed, or feedstuffs. These traceability approaches may also contribute to surveillance and prevention of unknown long-term effects on humans and environment.

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