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Fate of Transgenic *5-Enolpyruvyl-Shikimate-3-Phosphate Synthase (Cp4 Epsps)* Dna from Roundup Ready® Canola in Intestinal Epithelial Caco-2 Cells

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Abstract: We investigated the stability and fate of *5-enolpyruvyl-shikimate-3-phosphate synthase (cp4 epsps)* DNA from Roundup Ready® canola, in human intestinal Caco-2 cells *in vitro* and monitored the process at 0.5, 1, 2, 4 and 24 h. We found that 1.3 kb *cp4 epsps* transgenic DNA degraded rapidly and could not be detected by PCR in the culture media, cell washes or in DNA extracted from cells as early as 0.5 h of culture. However, transgenic fragments (278- and 270- bp) could be detected from culture media at all time points especially at higher concentrations (100 ng mL^{-1}). These fragments were detected from Caco-2 cell as early as 0.5 h and were consistently found at 1, 2, 4 and even at 24 h. The detection of transgene fragments from DNA extracted from cells was also observed at 4°C. Our results suggest that transgenic DNA fragments are associated with and possibly internalized by Caco-2 cells. To further support this observation, experiments were modified to include DNase I treatment and high salt washes. DNase I treatment removed all extracellular DNA; however DNA fragments were still detected in DNA extracted from cells. Thus 1.3 kb *cp4 epsps* is prone to rapid degradation but transgene fragments are cell-associated in human intestinal cells *in vitro*.

Key words: Caco-2 cells, *cp4 epsps*, DNA uptake, intestinal cells, Roundup Ready® canola

INTRODUCTION

The increasing use of Genetically Modified (GM) crops has been the topic of considerable scientific debate and public concern. The public wants to know if it is safe to consume foods that carry recombinant DNA. It is well-known that most nucleic acids contained in food are degraded in the upper digestive tract by the low pH in the stomach and active nucleases secreted into the Gastrointestinal (GI) tract. Despite this, ingested plant intrinsic and transgenic DNA of varying sizes persist and can be detected from the GI tract contents. For example, insecticidal-encoding c*ry9C* gene fragments (103 and 170 bp) were detected with varying frequencies from the rectal and cecal contents of pigs fed genetically modified maize (Chowdhury *et al*., 2003b). Similarly, fragments of the recombinant *cry1Ab* gene (110 bp and 437 bp) were detected from the gastrointestinal contents of Bt 11-fed pigs (Chowdhury *et al*., 2003a) and inconsistently from the rumen fluid of cattle (Chowdhury *et al*., 2004). Also, results from our laboratory indicate that although plant DNA is rapidly degraded under ruminal and intestinal conditions, the 1.3 kb transgene from Roundup Ready® canola meals and derived diets can be detected by PCR technique in the solid phase of the digesta in mixed ruminal culture *in vitro* for up to 8 h for canola meals and 4 h for mixed diets (Alexander *et al.* 2002). We further observed in *in vitro* cultures that the 1.3 kb transgene and fragments (300 and 527 bp) were stable for up to 10 min in ruminal fluid and for up to 4 h, depending on the fragment, in duodenal fluid at pH 3.2 (Alexander *et al*., 2004).

Roundup Ready® *Brassica napus* (canola) is tolerant to the herbicide glyphosate due to the presence of recombinant DNA from the CP4 strain of *Agrobacterium tumefaciens* encoding 5-enolpyruvyl-shikimate -3-phosphate synthase (*cp4 epsps*). Approximately 55% of the 48, 600 km^2 of canola grown in Canada were seeded to GM varieties in 2000 (Canola Council of Canada 2001) and in 2001, 256×10^6 kg of canola meal were used for ruminant animal production in Canada (Statistics Canada 2003). Considering the increased adoption of this oilseed crop, it is relevant to assess the fate of transgenic DNA arising from it. If, as mentioned above, foreign DNA of significant size is present in the intestine, it could theoretically be available for uptake by the endothelial/ epithelial cells and presumably by intestinal bacteria at points of high bacterial density such as the rumen or colon. It has been shown previously that oligonucleotides can enter mammalian cells (Zamecnik *et al*., 1986;

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Wickstrom *et al*., 1988) and intact oligonucleotides can be absorbed through the GI tract (Agrawal *et al*., 1995); however, there are very few studies on the fate of larger DNA fragments in the digestive tract. In one such study, phage DNA fed to mice was detected in several tissues and a fragment over 1.2 kb was re-cloned from total spleen DNA (Schubbert *et al*., 1997) suggesting that such large fragments of DNA from the gut can enter mammalian cells.

The goal of the present study was to assess the stability and cell-association of transgenic DNA *in vitro* and to establish the time duration that affects this process in cultured cells. Since there is not much information on the potential uptake of transgenic DNA by intestinal cells which is the first point of DNA uptake. These cells were chosen because monolayers of this polarized, human colon carcinoma cell line are considered to be a suitable *in vitro* model for the study of nutrient uptake and transport (Hidalgo *et al*., 1989) the cells spontaneously differentiate into an enterocyte-like phenotype in culture (Pinto *et al*., 1983). In this study, we chose purified DNA instead of mixed diets as a DNA source to mimic a situation where the DNA is released from food components and readily available for uptake by intestinal cells.

MATERIALS AND METHODS

Preparation of *cp4 epsps* DNA: Roundup Ready® canola seeds were obtained from Monsanto Company (St. Louis, MO, USA). The seeds were used for growing transgenic canola in the phytotron facility at the Lethbridge Research Centre under standard conditions. Genomic DNA was extracted from 21-d old leaves with the DNeasy® plant kit (Qiagen Inc., Mississauga, ON) and was used as template for PCR amplification of the 1.3 kb *cp4 epsps* gene using the forward primer F (5' TCA CGG TGC AAG CAG CCG TCC AGC 3') and the reverse primer R (5' TCA AGC AGC CTT AGT GTC GGA GAG TTC G 3'). PCR conditions used were 94°C for 5 min, 74°C for 5 min followed by 35 cycles of 94°C for 1 min, 74°C for 3 min and final extension at 72°C for 10 min. The amplified DNA (*cp4 epsps* DNA)was resolved on a 1% agarose gel and eluted using the Gel elution kit (Qiagen Inc.) with DNAse- and RNAse-free water, quantified using a spectrofluorometer (Bio-Rad Laboratories, Hercules, CA) and was diluted appropriately to various concentrations for supplementation in culture media for *in vitro* experiments.

Cell culture

Caco-2 cells: Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in Minimum Essential Medium with Earle's salts, L-glutamine and non-essential amino acids (Sigma-Aldrich Canada, Oakville, ON), supplemented with 18 mM sodium bicarbonate, 1.0 mM sodium pyruvate, 10 % fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT), 100 U mL^{-1} penicillin and 100: g μL^{-1} streptomycin (Gibco, Grand Island, NY). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was changed every second or third day. Stock cultures were passaged weekly by trypsinization.

For DNA uptake experiments, cells were cultured in 24-well plates (Nalge Nunc International, Rochester, NY). Confluent stock cultures were trypsinized and resuspended in culture medium. The viable cell density was measured with a haemacytometer using the trypan blue exclusion test and each well was seeded with 1×10^4 cells. The cells were used for experiments when confluent, which was usually after 12-15 d in culture.

Dna uptake experiments: The *cp4 epsps* DNA was added to Caco-2 cell monolayers in triplicate wells of 24-well plates. The DNA was included in 1 μL of medium to result in final concentrations of 0, 1, 10 or 100 100 100 ng mL^{-1}. The DNA was added to the cultures in a total volume of 10 FL. Cells were incubated for 0, 0.5, 1, 2, 4 or 24 h. After incubation, the culture media were collected for PCR analysis and the cell monolayers were washed five times with 1×Phosphate-Buffered Saline (PBS, for each wash,1 mL was added to each well, left for 3 min then removed) pre-warmed to room temperature. The fifth wash was also collected to check for the presence of *cp4 epsps* DNA by PCR. In a second set of experiments, DNAse I (1 U mL^{-1}; Roche Diagnostic, QC, Canada) was added to the first wash and the plates were incubated at 37°C for 30 min before collection of the first wash. Four additional washes were carried out and the fifth wash was collected. For both treatments (with and without DNAse) the rest of the procedure was the same: after the fifth wash, 150 μL of PBS was added to each well and the cells were scraped and collected. The wells were then rinsed with 50 μL of PBS which was pooled with the initial 150 μL PBS containing the cells. All experiments were performed in duplicate.

DNA extraction: Following DNA uptake experiments, DNA was extracted from collected Caco-2 cells using the DNeasy® Tissue Kit (Qiagen Inc.) according to the manufacturer's instructions for cultured animal cells. For DNA elution, the QIAamp spin column was Eluted (E1) with 100 μL of the Elution Buffer (EB) provided in the kit. The $A_{260:280}$ of each DNA sample was measured with a UV spectrophotometer (Ultrospec® 3000, Pharmacia Biotech, Cambridge, England) and used to calculate DNA concentrations to be used for PCR.

PCR amplification: In preliminary experiments, DNA was quantified from collected media, first and fifth washes and from Elutions (E1) following extractions. Several DNA concentrations were tested for PCR set up. We found that for the collected media, first and fifth wash, using 5 μL of sample as DNA template worked best and could amplify even trace amounts of added *cp4 epsps* DNA. We also attempted to extract the total DNA from media using DNeasy tissue kit but found that DNA yields upon extraction were too low to be reliably used in PCR. Therefore, a 5 μL aliquot of media was directly used for PCR. However, for DNA extracted from Caco-2, 250 ng of extracted DNA was found adequate and used as template in PCR. The limit of detection from cultures was found to be 90-100 pg.

Three primer sets were used to amplify regions in the *cp4 epsps* gene. The 1.3 kb complete transgene was amplified using the primers F/R as described in the previous section. Forward primer F_1 (5-CGT GGC TGA CTT GCG TG-3') and reverse primer R_1 (5'-CGT TAC CGA GAC CCT TAC C-3') amplified a 278-bp fragment (Fragment 1); forward primer F_2 (5'-TTG ATT GCG ATG AAG GTG AG-3') and reverse primer R_2 (5'-TCA AGC AGC CTT AGT GTC G -3') amplified a 270-bp fragment (Fragment 2). Fragments 1 and 2 were located within the *cp4 epsps* gene as shown in Fig. 1. The PCR mix consisted of (final concentrations): 1H PCR buffer, 1.5 μM $MgCl_2$, 0.2 mM dNTP mix, 0.5 FM each of forward and reverse primers and 2.5 U Platinum® *Taq* DNA Polymerase (Invitrogen, Burlington, ON). Each PCR set-up included a negative control (containing no template DNA) as well as a positive control consisting of 50 ng DNA template extracted from Roundup Ready® canola leaves. The PCR conditions on the PTC 100 thermocycler (M. J. Research Inc., Watertown, MA) for amplification of Fragments 1 and 2 were: 95°C for 5 min followed by 35 cycles of 94°C for 1 min, 58°C for 30 s, 72°C for 1 min and a final extension at 72°C for 10 min. PCR products (20 μL of each sample and 12.5 °L of the positive control) were loaded on a 1% agarose gel containing ethidium bromide in 1 H TAE buffer and resolved using standard procedures (Sambrook *et al*., 1989). Gene Ruler™ 100 bp DNA ladder Plus (MBI Fermentas, Burlington, ON) was used as the DNA marker. The gels were visualized on a trans-illuminator (Syngene, Frederick, MD) and photographed using standard procedures. Media and washes were subject to duplicate PCR reactions while DNA extracted from cells (Elution 1) was subjected to triplicate PCR reactions. The results were recorded as positive (+) or negative (-); in cases where results were inconsistent, PCR was repeated in duplicate to provide a clear positive or negative. This strategy is routinely used in our laboratory for transgene detection (Sharma *et al*., 2006).

Experimental design

Effect of the culture medium alone on the stability of cp4 epsps DNA: *Cp4 epsps* DNA (0, 1, 10 or 100 100 ng mL^{-1}) was added to 1 mL of culture medium in 24-well plates without cells and incubated for 0.5, 1, 2, 4 and 24 h. Media were collected and analysed for the presence of the 1.3 kb complete gene (primers F/R), Fragment 1 (primers F1/R1) and Fragment 2 (primers F2/R2).

Determination of the presence of the cp4 epsps and its fragments in DNA extracted from caco-2 cells: Confluent Caco-2 cells in 1 mL medium in 24-well plates were incubated with 0, 1, 10 or 100 100 ng mL^{-1} of *cp4 epsps* DNA for 0.5, 1, 2, 4 and 24 h at 37EC. The culture media were then collected, the cells washed, collected as described and DNA was extracted and used as template in PCR to amplify the 1.3 kb *cp4 epsps*, Fragments 1 and 2. PCR was also carried out on the media and 5th washes to detect the presence of the transgene in these samples.

Determination of the effect of DNAse I in the first wash on the stability of cp4 epsps in dna extracted from caco-2 cells: Confluent Caco-2 cells were incubated with 0, 1, 10 or 100 100 ng mL^{-1} of *cp4 epsps* DNA for various time points at 37°C. DNase I was added to the first wash as described previously (DNA uptake experiments) to degrade all the DNA in the media and that attached to the outer cell surface. Cells were collected as described, DNA was extracted and primers used to amplify Fragments 1 and 2. To ensure that DNase I was indeed active in PBS (the wash medium) and able to degrade all extracellular DNA, an experiment was performed in which *cp4 epsps* DNA (0, 1, 10 and 100 100 ng mL^{-1}) and DNase I (1 U mL^{-1}) were added simultaneously to PBS (1 mL) and incubated for 30 min at 37°C. The PBS was collected andanalysed by PCR for the presence of Fragments 1 and 2 after inactivation of DNase I.

Effects of low temperature and high salt washes on DNA uptake: Pre-cooled confluent Caco-2 cells were incubated with 0, 1, 10 or 100 100 ng mL^{-1} of *cp4 epsps* DNA for 2 h at 4°C. Cells were then washed once in pre-warmed PBS (1×) containing DNase I followed by four times in PBS and collected as described. DNA extracted from cells, media and wash samples were used to amplify Fragments 1 and 2. To verify whether the 4°C treatment affected cell viability, cells were incubated at 4°C as described, trypsinized and then subjected to the trypan blue exclusion assay.

In an another experiment, Caco-2 cells were incubated with 0 or 100 100 ng mL^{-1} of *cp4 epsps* DNA at 37EC for 2h. Cells were washed once in PBS (control) or with PBS containing 0.3, 0.5 or 1 M NaCl for 10 min. Cells were then

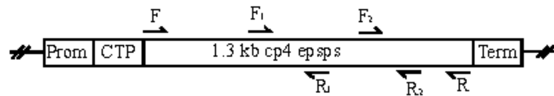

Fig. 1: Schematic representation of the 1.3 kb *cp4 5-enolpyruvyl shikimate-3-phosphate synthase (cp4 epsps)* construct showing location of the designed primers and the respective fragments amplified. The primers F and R amplify the 1.3 kb gene, primers F_1/R_1 amplify a 278 bp fragment (Fragment F1) and primers F_2/R_2 amplify 270 bp fragment (Fragment F2). Prom refers to the promoter, Term refers to the terminator region and CTP refers to the chloroplast transit peptide. Drawing not to scale

Table 1: Stability of Fragment 1 in the culture medium without cells for various periods of time

	DNA concentrations (100 ng mL^{-1})			
Time points (h)	0	1	10	100
0.5	-	+	+	+
1	-	+	+	+
2	-	+	+	+
4	-	+	+	+
24	-	-	-	+

+ = Present, - = Absent

washed four times in PBS and collected as described. DNA was extracted for amplification of Fragments 1 and 2. The effect of NaCl treatment on viability was also determined as described.

RESULTS

Effect of the culture medium alone on the stability of *cp4 epsps* DNA: The complete 1.3 kb *cp4 epsps* gene could not be detected in DNA extracted from confluent Caco-2 cells which were exposed to 1, 10 or 100 100 ng mL^{-1} of the DNA for 0.5, 1, 2, 4 and 24 h. As well, the transgene could not be detected in the corresponding media and washes (Fig. 1). These results prompted us to examine the stability of the transgene and its fragments in the culture medium over time to ascertain the stability of recombinant DNA in Caco-2 cell cultures. The *cp4 epsps* DNA was added to 1 mL of the culture medium in wells without cells; after 0.5 to 24 h of incubation, the media were used in PCR reactions. Fragment 1 could be detected in the culture medium after 0.5, 1, 2 and 4 h when added at concentrations of 1, 10 and 100 100 ng mL^{-1}; however, after 24 h, Fragment 1 was detected only when initially added at a higher concentration of 100 100 ng mL^{-1} (Table 1). Fragment 2 behaved similarly as Fragment 1 while the complete gene was never detected. This indicated that we should focus on smaller transgene fragments and that the DNA concentrations being used were adequate as they provided 2 extremes of low and high DNA concentrations.

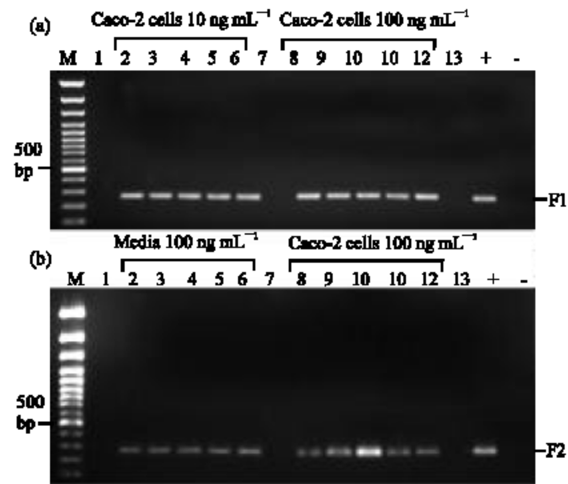

Fig. 2: Detection of Fragment 1 from Caco-2 cells from 0.5 to 24 h (at 10 and 100 ng mL^{-1} DNA concentrations as indicated) without DNase I treatment (Table 2). Lanes 2 and 8: 0.5 h; Lanes 3 and 9: 1 h; Lanes 4 and 10: 2 h; Lanes 5 and 11: 4 h; Lanes 6 and 12: 24 h. B). Detection of Fragment 2 from media (at 100 ng mL^{-1} DNA) and DNA from Caco-2 cells (at 100 ng mL^{-1} DNA) without DNase I treatment at 0.5 to 24 h (Table 2). Lanes 2 and 8: 0.5 h; Lanes 3 and 9: 1 h; Lanes 4 and 10: 2 h; Lanes 5 and 11: 4 h; Lanes 6 and 12: 24 h. For both the panels, Lanes M: 100 bp DNA Ladder Plus ; Lane + : positive control for PCR (DNA extracted from leaves of Roundup Ready® canola); Lane - : negative control (no DNA template in PCR); Lanes 1, 7 and 13: blank

Determination of the presence of the *cp4 epsps* and fragments in DNA extracted from caco-2 cells: In contrast to the 1.3 kb transgene, Fragment 1 was detected in DNA extracted from cells incubated in the presence of all DNA concentrations for 0.5 to 2 h (Table 2). When the cells were incubated with *cp4 epsps* DNA for 4 or 24 h, only the media and DNA samples from the wells containing 10 or 100 100 ng mL^{-1} DNA were positive for Fragment 1. A representative gel for the DNA extracted from Caco-2 cells at 10 and 100 100 ng mL^{-1} is shown in Fig. 2a, indicating that Fragment 1 is amplified from Caco-2 cell DNA at the indicated time points. Fragment 2 was detected in a similar pattern as Fragment 1 and tested positive in the media and Caco-2 cells even at 1 100 ng mL^{-1} DNA concentration, up to 4 h incubation (Table 2). Figure 2b shows a representative gel where Fragment 2 is

Table 2: Detection of Fragments 1 and 2 in the culture media, fifth wash and DNA extracted from Caco-2 cells after incubation with different concentrations of *cp4 epsps* DNA for various periods of time

	Media DNA concentrations (ng mL^{-1})				5th wash DNA concentrations (ng mL^{-1})				Caco-2 cell DNA DNA concentrations (ng mL^{-1})			
Time points (h)	0	1	10	100	0	1	10	100	0	1	10	100
Fragment 1												
0.5	-	+	+	+	-	-	-	-	-	+	+	+
1	-	+	+	+	-	-	-	-	-	+	+	+
2	-	+	+	+	-	-	-	-	-	+	+	+
4	-	-	+	+	-	n/a	n/a	n/a	-	-	+	+
24	-	-	+	+	-	-	-	-	-	-	+	+
Fragment 2												
0.5	-	-	+	+	-	-	-	-	-	+	+	+
1	-	+	+	+	-	-	-	-	-	+	+	+
2	-	+	+	+	-	-	-	n/a	-	+	+	+
4	-	-	+	+	-	-	-	n/a	-	+	+	+
24	-	-	+	+	-	-	-	.	-	-	+	+

+ = Present, - = Absent, n/a = not analyzed

Table 3: Detection of Fragments 1 and 2 in the culture media, fifth wash and DNA extracted from Caco-2 cells after incubating with different concentrations of *cp4 epsps* DNA for various periods of time. DNAse I was included in the first wash

Time points (h)	Media DNA concentrations (ng mL^{-1}) 0	1	10	100	Caco-2 cell DNA DNA concentrations (ng mL^{-1}) 0	1	10	100
Fragment 1								
0.5	-	+	+	+	-	+	+	+
1	-	-	+	+	-	+	+	+
2	-	+	+	+	-	+	+	+
4	-	-	-	+	-	-	+	+
24	-	-	-	+	-	-	-	+
Fragment 2								
0.5	-	+	+	+	-	+	+	+
1	-	-	-	+	-	+	+	+
2	-	-	+	+	-	+	+	+
4	-	-	-	+	-	-	-	+
24	-	-	-	-	-	-	-	+

+ = Present, - = Absent

detected from the media as well as the Caco-2 cell DNA at 100 100 ng mL^{-1} DNA concentrations from 0.5 to 24 h. These fragments were cloned and sequenced and found to have 98 % homology with the *cp4 epsps*.

Determination of the effect of DNase I in the first wash on the stability of *cp4 epsps* fragments in DNA extracted from caco-2 cells: To determine whether the *cp4 epsps* fragments detected in DNA extracted from Caco-2 cells had been internalized by the cells or were merely attached to cell membranes, we examined the effects of including DNase I in the first wash post-incubation on the presence of *cp4 epsps* fragments in DNA extracted from cells. Table 3 shows that the pattern of fragment detection is very similar to the one obtained previously (without DNase I treatment) except that after 24 h, only the DNA from cells exposed to 100 100 ng mL^{-1} was positive for Fragments 1 and 2. In this experiment, Fragments 1 and 2 were not detected in the media after 4 and 24 h, respectively, at the lower DNA concentrations tested. When we investigated the activity of DNase I under the wash conditions (in presence of PBS for 30 min at 37EC) we found DNase I to be active in PBS and no *cp4 epsps* DNA was detected by PCR after incubation of 0, 1, 10 and 100 100 ng mL^{-1} *cp4 epsps* DNA in PBS + DNase I for 30 min. These results indicate that DNase I could degrade all extracellular DNA within 30 min of incubation even when the DNA was at a high concentration of 100 100 ng mL^{-1}, indicating that if DNA is detected from Caco-2 cells it is probably the result of internalization.

Effects of low temperature and high salt washes on DNA uptake: The results of the previous experiments indicate that the DNA fragments being detected from Caco-2 cells is probably due to internalization of DNA. We performed uptake experiments at 4EC to observe if this supposed internalization is by passive diffusion across the cell membrane. Fragments 1 and 2 were detected in DNA extracted from Caco-2 cells when the cells had been incubated at 4EC with 10 or 100 100 ng mL^{-1} *cp4 epsps* DNA for 2 h (Table 4). Even though the viability of cells incubated at 4°C, as determined by the trypan blue exclusion assay, was not significantly different from the viability of cells kept at 37°C.

Furthermore, cells were incubated with 100 100 ng mL^{-1} *cp4 epsps* DNA for 2 h and the first wash contained NaCl at varying concentrations (0-1 M) to determine whether *cp4 epsps* DNA being detected from Caco-2 cells could be due to its extracellular association with cell membranes through ionic interactions. Fragment 1 was no longer detected in DNA from Caco-2 cells incubated with *cp4 epsps* DNA when washed with 1M NaCl (Table 5). Detection of Fragment 2 was found to be more sensitive to the concentration of NaCl since it was no longer detected in DNA extracted from Caco-2 cells incubated with *cp4 epsps* DNA and washed with either 0.5 or 1 M NaCl (Table 5). Fragment 1 and 2 could be amplified from the media but could not be detected in the fifth wash. The immediate viability of cells exposed to NaCl at all concentrations was not significantly different from the viability of cells washed in normal PBS.

Table 4: Detection of Fragments 1 and 2 in the culture media, fifth wash and DNA extracted from Caco-2 cells after culture with different concentrations of *cp4 epsps* DNA for 2 h at 4°C

	Media DNA concentrations (ng mL^{-1})				5th wash DNA concentrations (ng mL^{-1})				Caco-2 cell DNA DNA concentrations (ng mL^{-1})			
	0	1	10	100	0	1	10	100	0	1	10	100
Fragment 1	-	-	+	+	-	-	-	-	-	-	+	+
Fragment 2	-	+	+	+	-	-	-	-	-	-	+	+

- = Absent, + = Present

Table 5: Detection of Fragments 1 and 2 in the culture media, fifth wash and DNA extracted from Caco-2 cells after incubating with 0 and 100 100 ng mL^{-1} *cp4 epsps* DNA for 2 h. The first wash contained NaCl from 0 to 1 M

NaCl concentration (M)	Media DNA concentrations (ng mL^{-1})		Caco-2 cell DNA DNA concentrations (ng mL^{-1})	
	0	100	0	100
Fragment 1	-	+	-	+
0	-	+	-	+
0.3	-	+	-	+
0.5	-	+	-	+
1.0	-	+	-	+
Fragment 2				
0	-	+	-	+
0.3	-	+	-	+
0.5	-	+	-	+
1.0	-	+	-	+

-, Absent, +, Present

DISCUSSION

Gastrointestinal epithelial cells are in constant contact with DNA derived from food and provide a route for uptake of feed-associated DNA by absorption. The determination of the extent to which this happens is relevant as the use of genetically modified food and animal feed has been the subject of considerable debate and public concern. In the present study, we used Caco-2 cells to study the interaction and stability of 1.3 kb *cp4 epsps* DNA in cell culture. Although a few studies have investigated the transfer of bacterial DNA to cultured cells (Grillot-Courvalin *et al*., 1998, 2002) and it has been demonstrated that oligonucleotides can enter mammalian cells (Zamecnik *et al*., 1986; Wickstrom *et al*., 1988) to date few studies have examined the fate of larger DNA molecules in the presence of cultured intestinal cells, when the DNA is present in naked form. Furthermore, under *in vitro* cell culture conditions, the fate of large versus small DNA fragments has not been elucidated. Although M13 phage DNA fed to mice was detected in several tissues and spleen indicating that DNA from food can enter the blood stream through uptake by intestinal cells (Schubbert *et al*., 1997).

In the present study, even though the complete 1.3 kb DNA was supplied for uptake, it could not be detected in the culture media, in washes after all incubation times and in DNA extracted from cells. Similarly, the transgene could not be detected in the culture medium without cells, but shorter fragments could be detected in the culture medium for at least 24 h possibly due to mild nuclease activity of the serum included in culture media. The predominant nuclease activity in fetal calf serum has been reported to be 3' exonuclease (Shaw *et al*. 1991). When serum was omitted in the experiment without cells, trace amounts of the complete fragment could be detected up to 4 h further supporting the serum nuclease activity. Similarity in a study on the interaction of oligonucleotides with Caco-2 cells, genuine nuclease activity was not observed for at least 30 min in the presence of the cells cultured in a medium without serum (Beck *et al*., 1996).

The detection of Fragments 1 and 2 from Caco-2 cells in the first experiment (Table 2) seems to be due to internalization of these fragments or due to these fragments being bound to cell membranes. To discriminate between these two possibilities, we modified the initial experiment in three different ways:

- DNase I was added to the first wash following incubation of the cells with DNA.
- The uptake experiment was performed at 4°C instead of 37°C
- After co-incubation of cells with DNA, high salt washes were performed.

Our results clearly indicated that when DNase I was included in the first wash, *cp4 epsps* DNA fragments could still be detected from Caco-2 cells previously cultured in the presence of the transgene for 0.5 to 24 h (only highest concentration detected at 24 h). Since DNase I was active in the conditions of this study, these results indicate that some of the *cp4 epsps* DNA fragments could be possibly internalized by the cells rather than bound to the cell membranes. F1 and F2 were still detected from Caco-2 cells when the incubation was performed at 4°C (and DNase I was added to the first wash). At this temperature, cells are unable to take up material by an active mechanism such as endocytosis; therefore, the DNA could have been internalized by passive diffusion across the cell membrane. In a study of interaction of methylphosphonate oligonucleotides with Caco-2 cells, internalization was generally much higher at 37°C than at 4°C. Moreover, in that study, after incubation at 37°C, the bulk of cell-associated oligonucleotides was refractory to removal by low pH or trypsin, while after incubation at 4°C, almost all cell-associated material could be removed by these treatments (Shoji *et al*., 1991).

That study further indicated that the rhodamine-labeled methylphosphonate oligonucleotides were partially co-distributed with FITC-dextran, a marker for endocytotic vesicles and lysosomes, indicating that some oligos were internalized by the same endocytotic pathway as dextran. Other studies have also shown that unmodified oligonucleotides can enter cells by endocytosis (Loke *et al.*, 1989; Yakubov *et al.*, 1989; Nakai *et al.*, 1996) indicating that transport mechanism of oligonucleotides, small DNA fragments and larger DNA molecules such as the ones used in the present study could be quite different.

A high NaCl concentration would be expected to dislodge DNA attached to cell membranes via ionic interactions; therefore, if DNA was internalized, it would be expected to still be detected in total DNA extracted from the cells after a wash with a high salt concentration. In the present study, *cp4 epsps* DNA could still be detected in DNA extracted from Caco-2 cells after washes with 0.3 and 0.5 M NaCl; however, no DNA fragments were detected upon washing with 1 M NaCl. These results indicate that the DNA is internalized and thus detectable on extraction from cells upon washing with low salt concentration (0.3 M). However, why no DNA is detected from cells upon 1 M NaCl wash for Fragment 1 and at 0.5 and 1M NaCl for Fragment 2 is not clear. Amplification of these samples was repeated three times and the same result was obtained; the presence of PCR inhibitors was also ruled out. A previous report indicated that sodium chloride washes post-incubation could remove up to 68% of the cell-associated oligonucleotides with the greatest amount of removal with 1 M (Beck *et al.*, 1996). However, in that study no PCR was performed to be able to ascertain the effect of NaCl washes on PCRability from cells.

Based on the present study, we observed that small DNA fragments are more likely to be cell-associated with the intestinal cells. However, based on the literature even if transgenic DNA of significant sizes can be internalized by intestinal cells, a number of factors would affect the stability and integration of such DNA molecules within the cells over time. One of the consideration being, DNA would have to resist the various conditions of the digestive tract that have a degradative effect (low pH, pancreatic DNases). In general, studies indicated that only a small portion of transgenic DNA can survive passage through the gastrointestinal tract. For example, an *in vitro* study that simulated the mammalian stomach and small bowel showed that 4% of the transgenes in GM soya and maize survived this treatment (Martin-Orúe *et al.* 2002). Further, a study with human ileostomists showed that although a small proportion of transgenic DNA survived passage through the stomach and small intestine, all the transgenic DNA was degraded within the colon (Netherwood *et al.*, 2004). Interestingly, in that study, there was some evidence of low-frequency of gene transfer from GM soya to the microflora of the small bowel prior to sampling. It is such concerns about the probability of transfer of DNA from GM food either to intestinal bacterial cells or human intestinal cells lining the GI tract that is prompting studies to address the fate of food DNA in the GI tract.

CONCLUSION

Our results indicate that smaller transgenic DNA fragments are stable in cell cultures up to at least 24 h at higher concentrations of 10 and 100 100 ng mL^{-1}. These fragments are detected from Caco-2 cell suggesting their probable uptake by the cells however, detection due to association with cell membranes can not be ruled out.

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