

DNA uptake inn mammalian cell cultures

By

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ACKNOWLEDGMENTS

From October 2008 to July 2009 I had the great fortune of being a part of the Microbiology group in the Department of Pharmacy, University of Tromsø and GenØk working with this master thesis. I would first and foremost like to thank my 4 supervisors. The main man and super professor himself Kaare M Nielsen, for his patience, encouraging words and precise and thorough guidance through this work. A huge thank you to Idun M Grønsberg and Ane LG Utne for so much help with everything during my work. Thank you for always being there with explanations and good help during my lab work and writing. Thank you also to Terje Traavik at GenØk.

Thanks to Anne Hilde Conradi for helping me with all the technical things in the microbiology lab. And thank you to the rest of the Microbiology group. It has been very enjoyable and a good learning experience to participate in meetings and discussions with you. Klaus Harms, thank you for your help and tips in the lab. Thank you to the people at GenØk. Signy Bendiksen, Connie Tümmeler and Endre Steigum for your help with important things in the lab. A large thank you to Lise Nordgård for helping me so much with the literature needed for this thesis.

Hanne Kildalsen , Rune Hogseth, Hilde Ljones and Jury Kiselev in the Pharmacology group at UiT for all their guidance in the cell lab.

A very special thanks to my good friend, lab partner and fellow student Viktoria Emily Rasmussen. Thank you for all the support during our time here at the University. Could not have made it without you.

Thank you to Wenche Olsen, Heidi M Eriksen, Chipu Tendeland & Elisabeth Weibust for all help and the fun times shared in our “office”

Kenneth thank you for always being encouraging and for all the fun had during this time.

Annbjørg Susanna Hætta.

July 2009, Tromsø,

ABSTRACT

It has long been held in view that DNA present in food is confined to, and becomes rapidly degraded in the gastrointestinal tract of mammals. This assumption has been shown to be accurate for the majority of DNA in food entering the gastrointestinal tract, but many studies have shown that digestion is not 100 %. DNA is a stable molecule that can survive the conditions in the gastrointestinal tract, and from here a possible uptake into cells, bloodstream and other organs has also been shown to happen. If taken up by cells there is also the possibility of the foreign DNA to be integrated into the hosts own chromosome.

Integration of foreign DNA in mammalian cells is probably a very low frequency event that makes the experimental detection of such events *in vivo* almost impossible with current available methodology.

This study uses an intestinal enterocyte like cell line (Caco-2) to test the possibility of spontaneous uptake and possible integration *in vitro*. The DNA used as marker is the plasmid construct pRc/CMV-SLT. It contains the Simian Virus 40 Large T-antigen which has been shown to have oncogenic potential under the control of the human cytomegalovirus immediate early promoter. If integrated into the mammalian cells, detectable changes would be observable by the changing of the cells morphology.

PCR targeting the large T-antigen segment was used to analyze DNA isolated from the cell cultures. The DNA from cells grown in the presence of pRc/CMV-SLT did give positive signals for large T-antigen fragments when amplified by PCR. However the method did not indicate whether the DNA had in fact passed into the cells and been integrated. For this the usual PCR is not accurate enough. No visual changes in the cultured cells morphology were observed during the study.

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ABBREVIATIONS

ATCC	American type culture collection
Bp	Base pairs
CaCl ₂	Calcium chloride
cDNA	Complimentary Deoxyribonucleic acid
DNA	Deoxyribonucleic acid
dsDNA	Double stranded Deoxyribonucleic acid
DMSO	Dimethyl Sulfoxide
EtBr	Ethidium Bromide
FBS	Fetal Bovine Serum
FISH	Fluorescent <i>in situ</i> hybridization
GFP	Green fluorescent protein
GIT	Gastrointestinal tract
JC virus	John Cunningham virus
LB	Luria Broth Base
LBA	Luria Broth Agar
LBamp ¹⁰⁰	LB amended with Ampicillin, 100 µg/ml
LAF	Laminar air flow
MEME	Minimum Essential Medium Eagle
MgCl ₂	Magnesium chloride
NaCl	Sodium chloride
NaOH	Sodium hydroxide
TAE	Tris-acetate-EDTA
PBS	Phosphate buffered saline
PBSA	Phosphate buffered saline added albumin
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SV40	Simian Virus 40

1. INTRODUCTION

1.1 Background

DNA ingested through food degrades as it passes through the digestive system of mammals. However it is believed that not all 100% of this ingested DNA is degraded, but some of it may be taken up by the organism. In our diets we ingest DNA from animal muscle tissues in high concentrations and also plant DNA, but this in lower concentrations. The calculated average amount of this ingested DNA is 0.1 – 1 g / person / day (Doerfler *et al.*, 1998).

Studies have shown that feed ingested DNA can be found transiently circulating in the bloodstream (Schubbert *et al.*, 1994). By feeding laboratory mice with circular and linearized DNA, and testing their blood up to 7 hours after feeding, DNA was found circulating in the bloodstream. Analyzed by Southern or dot blot hybridization, the size of fragments detected ranged from < 200 – 400 base pairs (bp). But in other studies fragments up to 1700 bp have been detected (Pakla-Santini *et al.*, (2003).

It is believed that the foreign DNA may come in contact with cells in various organs and that it might be integrated into the host's chromosome. In one study it was found the DNA fed to mice could later be traced covalently linked to the mouse's own DNA, indicating an integration event (Schubbert *et al.*, 1997). The DNA fed was traced in peripheral leukocytes by PCR, and located by fluorescent *in situ* hybridization (FISH) in white blood cells, spleen or liver cells of the mice. By FISH they also detected the fed DNA in the columnar epithelial cells, in the leukocytes in Peyer's patches of the cecum wall, in liver cells, and in B cells, T cells and macrophages from spleen. These findings suggest a possible entry way of the DNA into the mice to be through the intestinal wall. By the Peyer's patches to peripheral blood leukocytes and further to other organs. Both studies showed that a very small amount survived the gastrointestinal tract, (about 1-2%), and that an even smaller amount reached the bloodstream (approximately 0.1%).

Another study fed pregnant mice with foreign DNA for 1-2 weeks during pregnancy. Here the foreign DNA was found in the offspring (Shubbert *et al.*, 1998). The fragments of the orally ingested DNA (size around 830 bp) were found in cell clusters in several organs of the fetuses and new born mice by FISH. The patchy distribution of the foreign DNA containing cells in the various organs suggested that the transfer had occurred transplacentally and not by germ line transmission. All these studies suggest that the gastrointestinal tract was in fact a highly likely further entry way for foreign DNA into the host.

The gastrointestinal tract

The most common site for uptake of foreign DNA is the gastrointestinal tract (GIT) of mammals. The human lower gastrointestinal tract consists of the small and large intestine. The small intestine is the site of most of the digestion and absorption of nutrients. It has a large surface area and is almost always in contact with degraded foods which contain large amounts of foreign DNA. The food from the stomach is further degraded in the small intestine by enzymes. The transit time for foods in humans is approximately 30 hours and the turn over of epithelial cells is relatively high. Epithelial cells lining the intestine, enterocytes, are the transporters of molecules across the intestinal wall. The large intestine has no function as a degrader or transporter of nutrients, as it absorbs water and disposes of the abundant materials which the body has no use of.

DNA stability in the GIT

DNA can survive extreme conditions in the GIT as it is a stable molecule. Ingested DNA from feed/food components is in the gut by mastication broken down into small fragments by acid hydrolysis and gastro-intestinal enzymatic activity (involving DNase I and DNase II) (Mazza *et al.*, 2004). In the GIT there is a large reservoir of microorganisms. The bacteria in it self can be a source for foreign DNA transferring into the host's cells. Cultured human cells are shown to take up bacterial DNA, although only transiently, so integration is questionable (Anker *et al.*, 2004). In mice fed soybeans with a specific rubisco gene, the DNA survived up to 121 h in the GIT

(Doerfler *et al.*, 2001). The same study concluded that the foreign DNA fragments that might survive passage through the GIT are believed to be distributed into the organism via the blood and/or lymph system with white cells as transport vectors.

In a study by Neterwood *et al.*, (2004) human ileostomy patients (large intestine is surgically removed) were fed genetically modified soya beans. Transgenes from the soy beans was shown to survive in the upper GIT, but was fully degraded by the time it passed through the large intestine. The referenced studies above have shown that foreign DNA can in fact survive passage through the upper GIT and be taken up and further find its way to cells in the living organism. The mechanism of this possible uptake is not known. But Schubert *et al.*, (1997) believed that in mice the DNA may have entered the M cells and leukocytes in the Peyer's patches.

Uptake of DNA in mammalian cells

It is believed that mammalian cells can spontaneously take up foreign DNA. And if it escapes degradation inside the cell and reaches the nucleus, it is speculated that it may be integrated into their genomes. How is the DNA taken up by the cells? No mechanism for how the uptake happens is accurately proven. But there are several suggested mechanisms as to how DNA is taken up by cells.

When foreign DNA has crossed the GIT barrier, and then further made it into circulation. There is one more barrier for it to cross, the blood. Blood plasma has a high DNA hydrolyzing activity, with DNase I being the largest contributor for this, and therefore regulating the amount of foreign DNA circulating. But it also contains a number of proteins (immunoglobulins, albumin etc.) capable of binding the DNA and thus protecting it from further degradation (Vasslov *et al.*, 2007). DNA bound to proteins is thought to be another possible way for foreign DNA to enter cells. Basner-Tschakarjan *et al.*, (2004) identified ezrin and moesin as DNA-binding membrane-cytoskeleton linker proteins as being involved in uptake and trafficking of DNA. They did not identify any transmembrane DNA binding receptors. But ezrin and moesin are functionally associated with other membrane receptors.

The membrane of eukaryotic cells is a strong barrier which prevents passive diffusion of nucleic acids into the cell. A suggested mechanism for DNA uptake is endocytosis (figure 1).

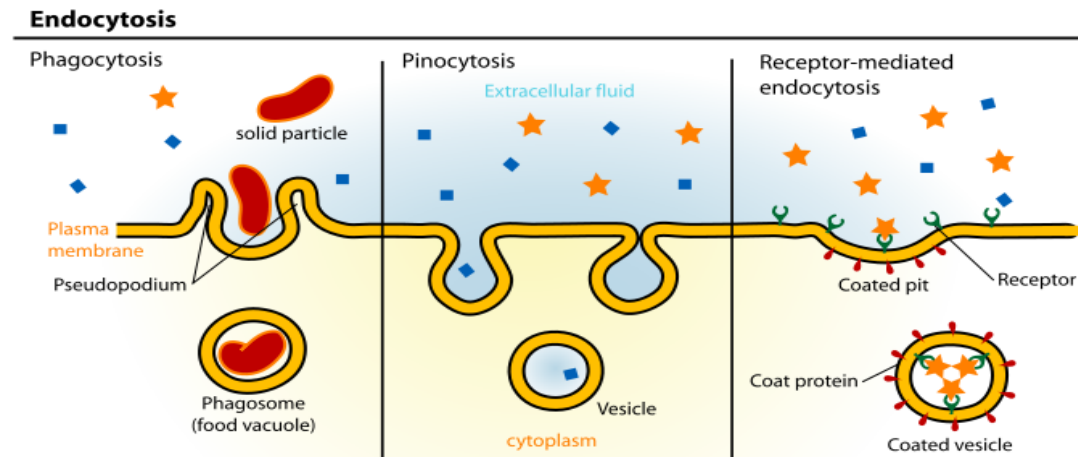


Figure 1: Three types of endocytosis. Figure from http://cellbiology.med.unsw.edu.au/units/images/endocytosis_types.png

Endocytosis is used by mammalian cells to internalize fluids, macromolecules and particles they need that can not pass into the cell otherwise. The three different types of endocytosis in the figure all have different way to take in the molecules. Phagocytosis is cell eating, where the cell engulfs macromolecules into a sealed of pocket (phagosome). Pinocytosis is cell drinking, and it takes up fluids and single molecules. These two forms are non specific and can also take up unwanted molecules. The third type of endocytosis is receptors-mediated.

Experimental studies have suggested the two latter to be a possible way for uptake of foreign DNA. They have further named proteins and receptors that can take part in nucleic acid internalization. The proteins isolated from cellular membranes and named (for example: Albumin, cytokeratins K1, Moesin and Ezrin), are already known to be cell-surface proteins. A specific nucleic acid binding receptor has been described by many researchers to be responsible for transportation of the nucleic acid

across the membrane and into the nucleus, but it still remains uncharacterized (Vasslov *et al.*, 2007).

Mammalian cells exposed to naked double stranded DNA (dsDNA) were shown in a study to be spontaneously internalized and expressed (Lehmann *et al.*, 2005). The study suggested an endocytotic uptake pathway for the dsDNA. This process was believed to be cell type dependent suggesting that specific cells may preferentially be susceptible for naked dsDNA.

Consequences of integrated DNA

But what happens if the DNA is taken up, integrated and further expressed in the cells? A possible result if foreign DNA is integrated into cellular DNA is that it may have the potential of tumor induction (Kurth, 1998). Kurth further describes the possibilities of foreign DNA integration to rarely have any harmful effects on the host. But if integrated there are several possible consequences. The 1998 article describes 8 possible outcomes of foreign DNA (chromosomal) integration. If integrated into a biologically inert region there would be no expression of the integrated DNA. But if integrated into a biologically active region, this could lead to stable expression. The stable expression could further be silenced (DNA methylation, mutation, excision). And an integration into vital cellular genes could lead to an inactivation of these and therefore cell death. Integrated foreign DNA could also lead to activation of protooncogenes and the creation of (tumor specific) fusion proteins or inactivation of tumor suppressor genes. Lastly a foreign DNA chromosomal integration could lead to chromosomal instability, the examples being DNA breaks, rearrangements or changes in methylation patterns.

1.2 Large tumor antigen

DNA tumor viruses depend on a host's replication machinery to further complete its own viral replication cycle. Cancer is shown in some cases to be associated with human polyomaviruses, such as JC virus, BK virus and simian virus 40 (SV40), (Caracciolo *et al.*, 2006).

SV40 has a small genome (5243 bp) and a well defined replication origin that depends only on its large tumor antigen (T-ag) protein for DNA replication. The viruses T-ag has many built-in biochemical factors needed for the production of further virus copies. These factors for instance regulate the timing of the infection cycle and repress the transcription of its own gene, and the initiation of the viral DNA replication (Fanning *et al.*, 1992). The large T-ag functions that SV40 needs for its replication are ATPase, DNA helicase and specific DNA binding activities. These functions help the replication of viral DNA by binding and promoting the unwinding of the SV40 replication origin (Ali *et al.*, 2001). The ability of large T-ag to bind and inactivate tumor suppressor proteins (such as pRB and p53) gives SV40 its oncogenic potential (Dhaene *et al.*, 1999). And it is this ability which gives the SV40 the possibility to extend and transform the cells.

A study done with human cells in culture (fibroblasts) suggest that large T-ag by forming stable complexes with the p53 suppressor gene extends the life span of the cell (Lin *et al.*, 1991). In a review by Croul *et al.*, (2003) SV40 is connected to human brain tumors. Sequences specific for this virus have been found in the glial and ependyma cells in brain tumors. Though the topic of the possibilities of SV40 to cause human cancer is contentious and has been debated for over 50 years in oncology, ever since SV40 was found to have contaminated polio vaccines given to millions of people during the 50's. Poulins *et al.*, (2007) review concludes that the research surrounding the human oncogenic potential of SV40 is poorly conducted. As the methodologies for detecting SV40 in human tumors are flawed and that to date these methods are improved, concluding that studies done on the subject have failed to to provide convincing evidence implicating SV40 as a human pathogen.

Because of these properties of the large T-ag it was used as foreign DNA in this study. This therefore makes it a good marker for uptake and integration of the added foreign DNA. The commercially available plasmid pRc/CMV by Invitrogen (Germany) was inserted with large (T-ag) segment from the Simian Virus 40 (SV40) between Not I and Apa I by Moens *et al.*, (Moens *et al.*, 1995). See figure 2.

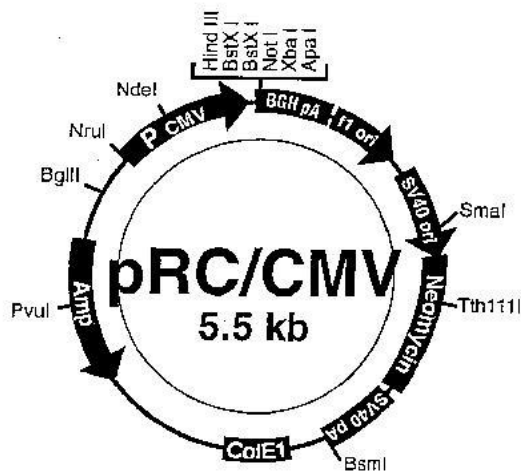


Figure 2: Plasmid pRc/CMV (by Invitrogen, Germany) used in this study with segment encoding large T-antigen inserted between Not I and Apa I. Figure from www.invitrogen.com

When inserted with the large T-ag segment, the plasmid pRc/CMV-SLT was constructed. The plasmid encodes the polyoma virus large tumor antigen (T-ag). This construct has a very strong oncogenic potential when under the control of the human cytomegalovirus immediate early promoter (Moens *et al.*, 2001).

1.3 Mammalian Cell cultures

Cell culture growing has been around since the 19th century, when it was discovered that isolated cells of living organisms could be maintained in solutions. In research the technique of maintaining living cells proved valuable when studying viruses. As an example of this, vaccines were developed growing viruses in cell cultures. A mammalian cell cultures can arise of any type of cell from a mammalian host. Cell

cultures derived from a primary source have a limited lifespan because after some divisions the cells reach a point where they stop dividing. Therefore cells have been modified to proliferate indefinitely.

The cell line used in this project was human epithelial cells. Caco-2 cells (American Type Culture Collection HTB-37; ATCC, USA) are colon cells from a 72 year old who was diseased with colorectal adenocarcinoma. A study by Jumarie *et al.*, (1991) concluded that the cells are very relevant *in vitro* models for studying differentiation and regulation of intestinal functions. Caco-2 cells are widely used for the study of intestinal drug transport, because of their morphological and functional similarities of intestinal enterocytes (Shah *et al.*, 2006). The cells have the ability to take up and transport ions, sugars and peptides when grown on plastic. On porous filters they have been able to exhibit polarized transport of bile acids and vitamin B₁₂. The passive and active transport properties of Caco-2 cell monolayers should therefore be similar to what is found *in vivo* (Hilgers *et al.*, 1990). For this study Caco-2 cells were chosen for these abilities of mimicking the gastrointestinal system.

1.4 Aims of study

Aims of study:

Determine whether foreign DNA when present in mammalian cell cultures can be spontaneously taken up by exposed cells, and detect possible rare events of integration of the DNA in the mammalian cell genome.

Main hypothesis:

Foreign DNA can be taken up and subsequently integrated and expressed by mammalian cell cultures, leading to detectable changes in cell morphology.

Specific objectives:

1. To grow epithelial cell cultures in the presence of isolated plasmid DNA carrying the construct pRc/CMV-SLT. This plasmid encodes the polyomavirus large T-antigen which has oncogenic potential under the control of the human cytomegalovirus immediate early promoter. In addition to plasmid DNA, PCR products carrying the same gene construct will be used to determine if different conformation has a role in uptake by the mammalian cells.
2. Uptake will be detected by using polymerase chain reaction to amplify isolated DNA from the cell cultures. And also by visually recording altered cell morphology.

2. RESULTS

2.1 Natural uptake of DNA in cell cultures

Exposure of DNA to mammalian cell cultures

Different amounts of circular and linear plasmid DNA were added to Caco-2 cell cultures to determine if there was a difference in the uptake between the two variations of DNA and the amounts of DNA. During the project, two different exposure time experiments were performed, short term exposure and long term exposure. In the short term exposure experiment, DNA amounts from 0,5 µg to 50 µg were added once and incubated for two days. In a long term exposure experiment, 50 µg was added daily for a period of 1 or 2 weeks. The cells were observed in a microscope daily to check for differences in behavior and appearance.

Effects of DNA concentration

Initial experiments were conducted with low amounts of plasmid DNA. From 0.5 µg (3.1, 3.2, 4.1 and 4.2 in figure 3), 1.0 µg (5.1, 5.2, 6.1 and 6.2 in figure 3) 2.5 µg (7.1, 7.2, 8.1 and 8.2 in figure 3) to 10 µg were added to cell cultures in two parallels and cells were left to grow for 2 days. Control cell parallels were not added any DNA (1.1, 1.2, 2.1 and 2.2 in figure 3) but otherwise treated the same way as parallels added DNA. As a positive control, parallels of cells were transfected with circular plasmid DNA. Cells were then harvested and the cell nuclei were isolated according to method described in Anker *et al.*, (2004). The isolated DNA was analyzed by polymerase chain reaction (PCR). To confirm that the isolated DNA from the nuclei was amplifiable (no PCR inhibitors) a PCR targeting the housekeeping gene β -actin (between 400-500 bp) was run for each sample. All isolated DNAs were amplifiable as shown in figure 3, with bands detected at the expected size (484 bp).

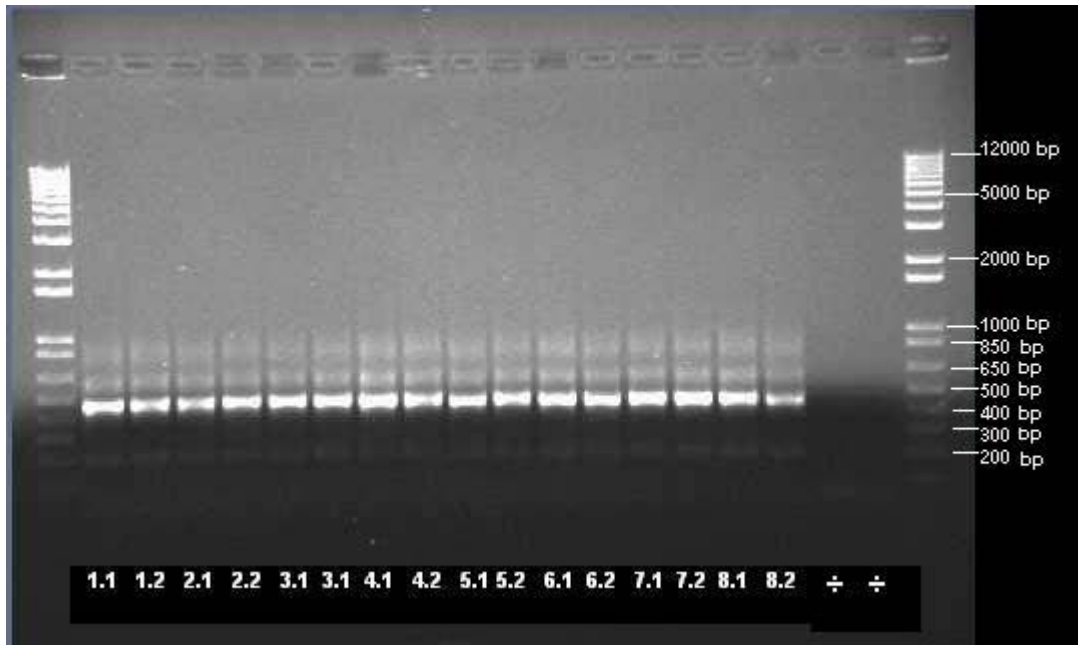


Figure 3: PCR targeting the β -actin housekeeping gene (size 484 bp) to check that the isolated DNA is amplifiable by PCR. Isolated from cell cultures added circular DNA from 0.5 μ g to 2,5 μ g (3.1 - 8.2 in figure) (controls added no DNA are 1.1 - 2.2 in figure). Each lane has a band at the expected size (between 400-500 bp) indicating that isolated DNA is amplifiable by PCR. Water template was run as negative PCR control. The ladder is 1 kb plus (Invitrogen, Germany).

Next a PCR targeting the large T-ag was run and this showed bands at the expected size (100 bp) in all samples where DNA was added to the cell cultures (results are shown in figure 4).

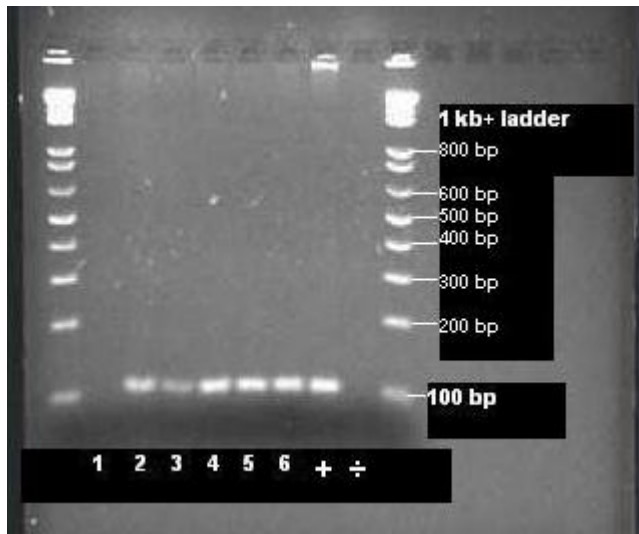


Figure 4: PCR targeting the large T-ag in DNA samples from the cell cultures incubated with circular DNA (fragment size 105 bp). Positive results are shown in lanes 2-4 where circular plasmid DNA was added to cell cultures, and lanes 5 & 6 which were from cell cultures transfected with circular plasmid DNA. Lane 1 = no DNA, lane 2 = 0.5 µg DNA, lane 3 = 1 µg DNA, 4 = 2.5 µg DNA, 5 = 2.5 µg large T-ag transfected parallel 1, 6 = 2.5 µg large T-ag transfected parallel 2, lane 7 positive PCR control (pRc/CMV-SLT plasmid) and lane 8 negative PCR control (water). (The ladder is 1 kb plus by Invitrogen, Germany).

All DNA samples were amplifiable, as shown in figure 3 with the targeting of the housekeeping β -actin gene. The PCR targeting the large T-ag showed that DNA in low concentrations added is possible to detect. Cell cultures transfected with pRc/CMV-SLT plasmid DNA also gave a positive PCR signal with a band appearing at the expected size (105 bp).

DNA conformation

To see if DNA conformation was a factor in uptake, circular and linear plasmid DNA was used. Linear DNA was made by PCR amplification of only the large T-ag segment including the promoter inserted in the pRp/CMV plasmid. The Caco -2 cell cultures were added 10 μ g, 25 μ g or 50 μ g DNA, in two parallels, and cells were left to grow for 2 days before DNA was isolated. Control samples were not added DNA but otherwise treated the same way. The PCR run targeting the housekeeping gene β -actin (size 484 bp) showed that DNA extracted from the cells was amplifiable (data not shown). The PCR targeting the large T-ag results for linear DNA show no bands at the expected size for the lowest amounts of DNA added, except for one band. Only in the cell cultures added 50 μ g of DNA, positive results were observed. One band appears in one of the parallels added 10 μ g DNA. Results are shown in figure 5.

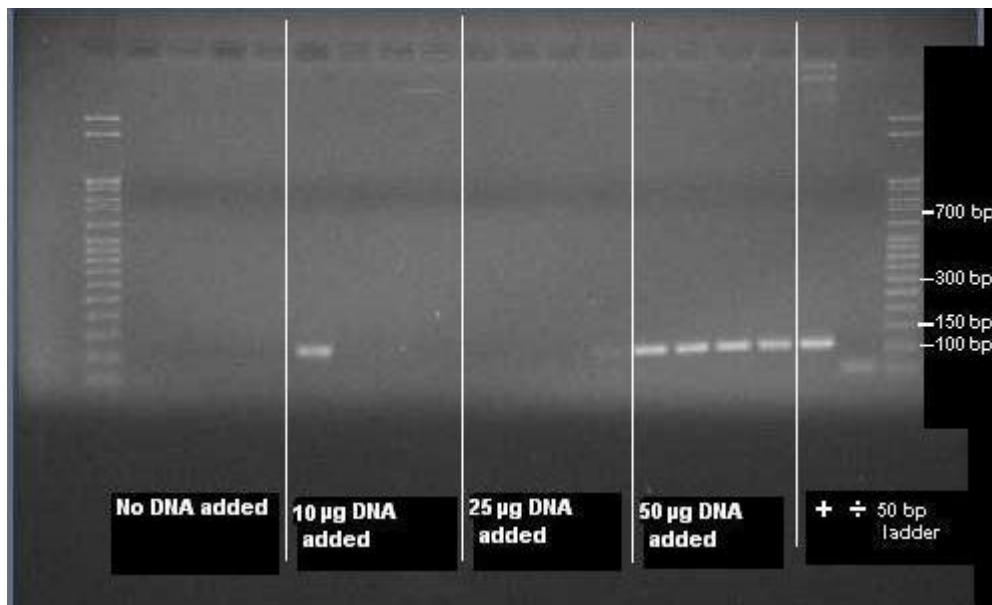


Figure 5: PCR targeting the large T-ag in DNA samples from the cell cultures incubated with linear plasmid DNA (fragment size 105 bp). Positive results are seen for cell cultures added 50 μ g of linear DNA and one parallel added 10 μ g DNA. Two parallels of each sample were run. Positive PCR control pRc/CMV-SLT plasmid and negative PCR control water. (50 bp ladder, Sigma-Aldrich, Germany).

The samples added circular plasmid DNA in the same amounts; show bands in all samples where DNA was added. Results are shown in figure 6.

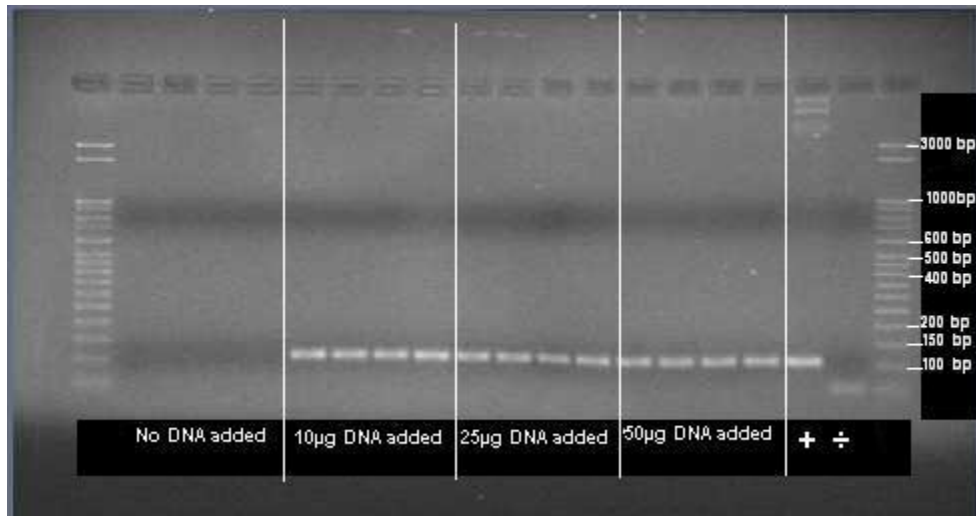


Figure 6: PCR targeting the large T-ag in cell cultures added circular plasmid DNA. Figure shows bands at expected size (100bp). Positive PCR control pRc/CMV-SLT plasmid and negative PCR control water. (50 bp ladder, Sigma-Aldrich, Germany).

The two different conformations of the DNA added to Caco-2 cell cultures show some differences in the parallels when detected with PCR targeting large T-ag. The parallels added the lowest amounts of linear plasmid DNA (10 and 25 µg) gave positive PCR results only in one parallel added 10µg. Only the parallels added the highest amount of linear plasmid DNA (50 µg) showed a positive result at the expected size (100 bp) in all parallels. The cell cultures added circular plasmid DNA all showed bands at the expected size.

Effect of exposure time

To see if length of exposure was significant for uptake of DNA, the DNA was added to Caco-2 cell cultures daily for a period of 1 or 2 weeks. The amount added of both circular plasmid DNA and linear DNA was 50 µg. The Caco-2 cell cultures were

added 50 µg of circular plasmid DNA daily for a period of 1 week before DNA was isolated from the cell nuclei. The PCR targeting the housekeeping gene β -actin (size 484 bp) showed that DNA isolated from the cells was amplifiable (data not shown). The PCR targeting the large T-ag showed positive results in the two parallels run of the cell culture added DNA (figure 7). The control culture was treated the same only no DNA was added.

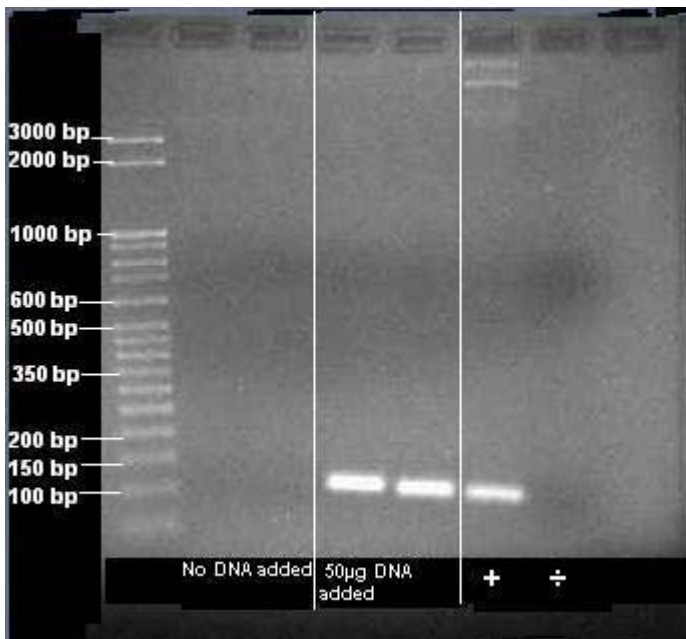


Figure 7: PCR targeting large T-ag (expected size 100 bp) of cell culture added 50 µg circular plasmid DNA (lanes 4 and 5) for a period of 1 week. Control samples: added no DNA (lane 2 and 3). Positive PCR control pRc/CMV-SLT plasmid and negative PCR control water. (50 bp ladder, Sigma-Aldrich, Germany).

To further explore possible the differences in uptake of circular plasmid DNA and linear DNA Caco-2 cell cultures were added 50 µg DNA in three parallels. One parallel was added circular DNA, and two parallels were added linear DNA. Addition of DNA was done daily for a period of 2 weeks. One additional cell culture parallel was used as control and was not added DNA. Every week the cells were split 1:4

with 3:4 being kept for analysis (DNA isolation) and 1:4 was let grow further. The third week, the cells were grown without any DNA added. The cell cultures were observed daily with a microscope to see if any physical changes occurred. At the end of the third week, the cell cultures were terminated and DNA isolated. The PCR targeting the housekeeping gene β -actin (size 484 bp) showed that DNA isolated from the cells was amplifiable (data not shown). The PCR targeting the large T-ag showed bands in all cell cultures where DNA was added (figure 8). There were no observations of physical changes of the cells during the experiment period (data not shown).

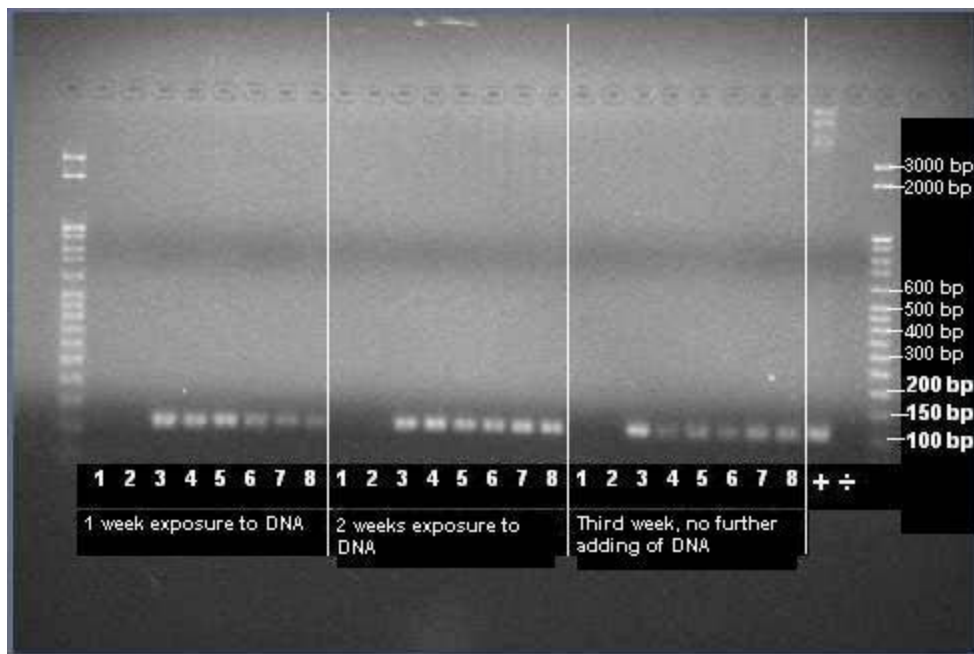


Figure 8: PCR targeting the large T-ag in cell cultures added DNA daily for a period of 2 weeks and let grow for three weeks total. Lanes 1 & 2 = Control (no DNA), lanes 3 & 4 = 50 μ g circular plasmid DNA added, lanes 5 & 6 = 50 μ g linear plasmid DNA added (parallel 1), lanes 7 & 8 = 50 μ g linear plasmid DNA added (parallel 2). Positive PCR control pRc/CMV-SLT plasmid and negative PCR control water. (50 bp ladder, Sigma-Aldrich, Germany).

Large T-ag was detected after 1 week of exposure to the cell cultures. When the cells were let grow for a week without further adding of DNA, there were still positive results in the PCR targeting the large T-ag. There is no difference in between circular and linear plasmid DNA. Both conformations gave positive PCR results.

Effect of DNase treatment

The PCR runs targeting the large T-ag indicated the presence of large T-ag in almost every cell culture where plasmid DNA was added, and also cell cultures where higher amounts of linear DNA were added indicating DNA uptake and transfer to nuclei. It was speculated to be a false positive result. So to see if this was the case cell cultures were added circular plasmid DNA for only a few seconds before DNA was isolated from the nuclei, allowing no time for uptake. When PCR targeting large T-ag was run with the isolated DNA samples it gave positive results in the samples added DNA and negative results for samples where no DNA was added (data not shown). Experiments with DNase treatment were therefore conducted to try to eliminate possible DNA attached to the cell nuclei membrane. Cell cultures were added 50 µg plasmid DNA before nuclei were harvested directly after addition (method described 5.3). The harvested cell nuclei were treated with DNase in various concentrations over different time periods (15, 30 and 60 minutes). DNA was isolated and PCR targeting the large T-ag was run of samples. The PCR results still showed positive bands indicating the DNase treatment had not been effective (data not shown), or that DNA was associated to cell surfaces rendering it protected from the nucleases.

Visualization of DNA uptake

During the periods were Caco-2 cell cultures were grown with DNA, the cell cultures were observed under microscope for any morphological changes. No morphological changes were observed in the Caco-2 cells added DNA, or in the control cell cultures added no DNA (data not shown). As a control for spontaneous DNA uptake, phrGFP-1 was added to Caco-2 cell cultures. This was done to see if cells would spontaneously take up foreign DNA and express it. In this case if phrGFP-1 was taken up naturally by cells they would be seen as green when visualized by fluorescent microscopy. These cells were observed under a fluorescent microscope to check for uptake. No fluorescent cells were observed in any of the cell cultures added phrGFP-1 (data not shown).

Caco-2 cells transfected with phrGFP-1 however, were possible to visualize by fluorescent microscope. The results are shown in figure 9 a/b.

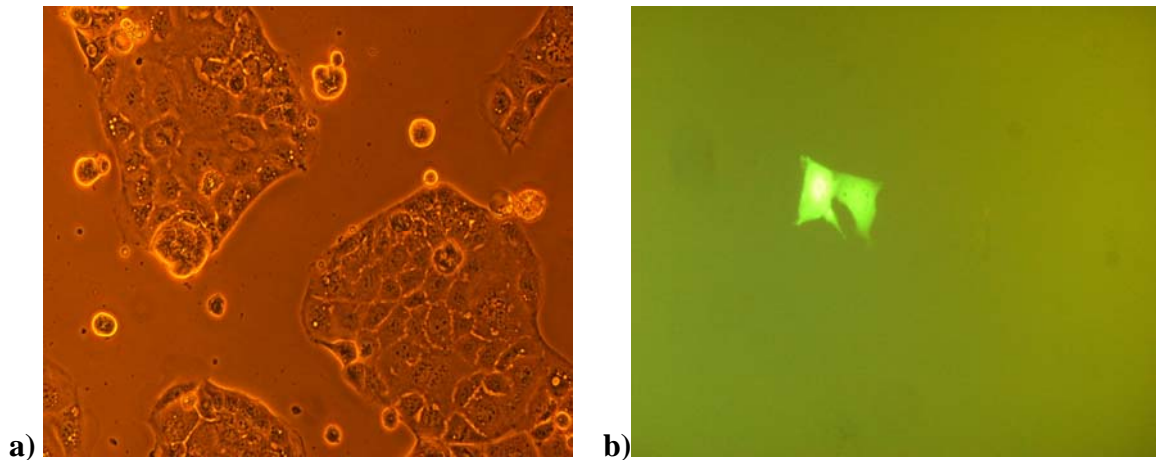


Figure 9 a/b : Caco-2 cells transfected with phrGFP-1. The phrGFP-1 is expressed in cells signaling green when visualized by fluorescent microscope (b).

Caco-2 cells transfected with pRc/CMV-SLT plasmid DNA showed signs of changes in morphology when viewed in a microscope 36 hours later (data not shown). The cells seemed to not thrive as well when transfected, as their normal round shape was changed to be a little more rougher around the edges. This was not observed with natural uptake cell cultures.

2.2 Artificial uptake of DNA in cell cultures by transfection

Expression of large T-ag in Caco-2 cell cultures

Immunostaining

To check if large T-ag could be expressed in the cell line used, three different staining methods were used. Cells were transfected with pRc/CMV-SLT plasmid DNA for 24 and 36 hours before staining was performed. Immunostaining was done with Sigmafast™ OPD (Sigma, Germany) as substrate. But the signal was too weak, so no staining could be observed (data not shown). Same procedure was done a second time with a different substrate (Immunopure® Metal Enhanced DAB (Pierce, USA)). Signal was also here too weak so no staining was observed (data not shown). Lastly a fluorescent staining substrate (AlexaFluor 488; goat anti-mouse IgG F (ab')₂, Invitrogen, Germany) was used and this time the cells were successfully stained. Green cells were observed under fluorescent microscope. The observations are shown in figures 10 a/b for 24 hour transfected cells and 11 a/b for 36 hour transfected cells.

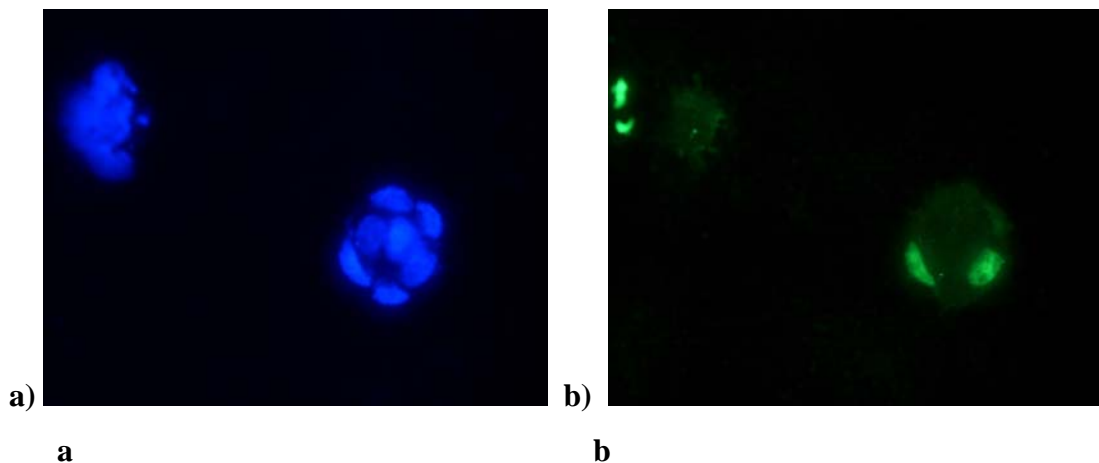


Figure 10 a/b: Caco-2 cells transfected 24 hours with pRc/CMV-SLT plasmid DNA. Large T-ag is expressed in cells signaling green when visualized by fluorescent microscope (b). Normal microscope view (a).

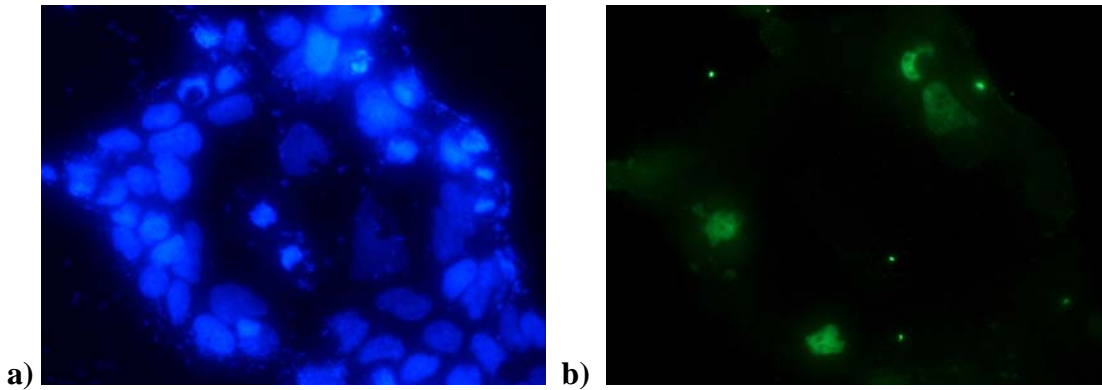


Figure 11 a/b: Caco Caco-2 cells transfected 36 hours with pRc/CMV-SLT plasmid DNA. Large T-ag is expressed in cells signaling green when visualized by fluorescent microscope (b). Normal microscope view (a).

The second method used to check expression of large T-ag was construction of cDNA from RNA isolated from cells transfected with pRc/CMV-SLT plasmid DNA. This was done by first transfecting Caco-2 cells for 24 hours or 48 hours with pRc/CMV-SLT plasmid DNA. A control sample was treated the same way only not transfected with any DNA. RNA was extracted from each sample, cDNA was made from RNA transcript. To check for presence of T-ag RNA transcripts, PCR targeting the large T-ag was performed on the cDNA samples. Bands appeared on gel when visualized. Results are shown in figure 12 and show bands at expected size in the transfected cell cultures (100 bp).

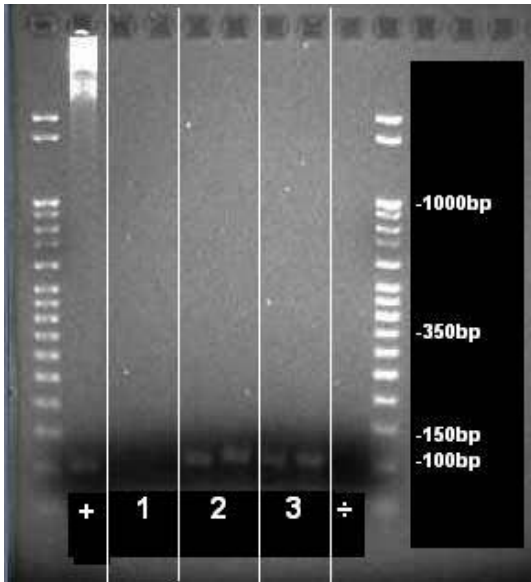


Figure 12: PCR targeting the large T-ag in DNA samples from Caco-2 cells transfected with pRc/CMV-SLT plasmid DNA. Figure showing positive bands in the cell cultures transfected with DNA for 24 hours (2 in figure) and 48 hours (3 in figure). cDNA was made by reverse transcriptase indicating that RNA for large T-ag was present in cells, and the PCR targeted the large T-ag in the cells. Lane 1: control cells, not transfected with DNA parallel 1 and 2. Positive PCR control pRc/CMV-SLT plasmid, and negative PCR control water. (50 bp ladder, Sigma-Aldrich, Germany).

2.3 Detection limits for the PCR reaction

As a control for how many DNA copies the PCR targeting the large T-ag could detect and amplify, a dilution series of plasmid DNA was made, and a PCR targeting the large T-ag was run for each dilution. First the DNA copy number in 1 μg of pRc/CMV-SLT was calculated using the formula from the article by Lee *et al.*, 2005 (approximate size of the plasmid 8500 bp). Dilutions were made 1000 folds, to contain 2.4×10^7 , 2.4×10^4 and 2.4×10^1 which was diluted down to 1 copy pr μl . The dilutions were then run with the PCR profile targeting large T-ag, with 1 μl of each dilution used as template. The first run showed bands down to the dilution with 2.4×10^4 DNA copies pr μl (data not shown). To see if it was possible to detect copies

lower than the 2.4×10^4 dilution, that dilution was then used to dilute 10 folds down to 2.4×10^3 , 2.4×10^2 , 2.4×10^1 and 1 copies pr μl . This was done to check if it would affect the result by being a more accurate dilution series. When PCR targeting large T-ag was run with these dilutions, it showed a weak band in the dilution containing $2,4 \times 10^3$ but not with fewer copies (results shown in figure 13).

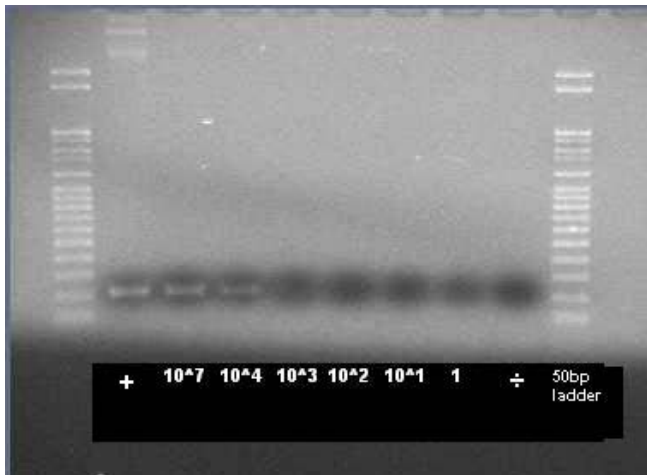


Figure 13: PCR targeting the large T-ag when the pRc/CMV-SLT plasmid DNA was diluted 10 folds down to 1 copy pr μl . Positive PCR control pRc/CMV-SLT plasmid, and negative PCR control water. (50 bp ladder, Sigma-Aldrich, Germany).

3. DISCUSSION

In this study the main objective was to see if foreign DNA could be taken up spontaneously by mammalian cells, and further integrated into the cells own DNA. As the GIT is very much in constant contact with foreign DNA, a possible uptake of this foreign DNA into a host could happen. From here the DNA can enter the bloodstream and further come in contact with other cells in a living organism. This is shown to happen by Schubbert *et al.*, (1994), where blood of mice fed circular and linearized DNA contained traces of the feed derived DNA. A possible uptake can also happen in cells further inside the organism. If the DNA survives the conditions within the cells and passes through to the nuclei, it could be integrated into the hosts DNA. The Caco-2 cells used in this study are shown to be good models for the uptake processes in the cells of GIT. Therefore they were chosen to be used together with DNA that has an oncogenic potential. If the oncogenic DNA is taken up, integrated and expressed by the cells, it could have the ability to transform the Caco-2 cells.

The Caco-2 cell cultures in this study were added circular and linear plasmid DNA of pRc/CMV-SLT in different concentrations, and using PCR as analysis targeting the large T-ag as a marker to locate the added DNA. The cells were incubated with the DNA for two days and up to two weeks. After the incubation period the cells were treated to harvest only the cell nuclei (method derived from Anker *et al.*, 2004, see section 5.3), and from the cell nuclei DNA was isolated and then analyzed by PCR. Low amounts of circular plasmid DNA added was detected using PCR targeting the large T-ag after only being incubated with the cells for two days. The same results were not seen for the same low amounts of linear plasmid DNA, only for higher amounts. Cells were observed during the study by microscope, but there were no changes observed in the physical appearance of the cells. The circular plasmid DNA was also transiently transfected into the Caco-2 cells. From visually inspecting the cells with microscopy it was possible to see that the cells did not thrive as well when they had undergone this process. In addition to observing natural uptake, the Caco-2 cells were also transiently transfected with the circular plasmid DNA. This was done to check if the cells were able to express the large T-ag protein, and also by visually inspecting them and look for physical changes. Different types of immunostainings were performed to check for expression of proteins. These showed positive results when stained with a fluorescent substrate. In the transfected cells RNA was collected and from this cDNA was synthesized. The PCR targeting the

large T-ag was positive for the cDNA samples indicating that the protein for large T-ag was transcribed in the cells.

Variations of the DNA concentration did show some differences from the lowest amounts added to the highest. The lowest amount of circular plasmid DNA added was 0.5 µg and this was detected by the PCR targeting the large T-ag. The lowest amounts of linear plasmid DNA added were 10 and 25 µg. Except for one positive signal in one of the parallels added 10 µg there were no other positive signals for the other parallels. Only when added 50 µg of linear plasmid DNA did the PCR detect in all the parallels. DNA conformation as discussed above also showed some differences in uptake. The circular plasmid DNA was always detected by the PCR, but the linear plasmid DNA was only detected in the highest amounts (50 µg DNA). Effects of the length of exposure did not show any differences. The PCR detected the large T-ag in cell cultures where they were incubated with circular plasmid DNA for seconds, days and weeks.

However, a positive PCR signal for plasmid and linear DNA of pRc/CMV-SLT does not necessarily indicate that the cells had taken up the foreign DNA. It is possible the DNA had associated itself on the outside of the cell. Anker *et al.*, 2003 showed that the foreign DNA could transitionally be found inside the cells. But like in their case when cells were incubated with DNA for mere seconds, there was a positive result seen then also. In this study it was experimented with different concentrations and time periods of DNase, to try and eliminate the possible DNA attached to the outside of the cell. This gave no results, as the PCR results of the cells when this was performed were still positive. There is a possibility of the foreign DNA to have attached itself to the cell by mechanisms the DNase could not have been able to break.

Visualization of changes in morphology was done by inspecting the cells during the experiments in a microscope. No observed changes were seen in the natural uptake cell cultures. Caco-2 cells transfected with circular plasmid DNA however did show some physical changes. The transfected cells did not seem to like the conditions they were submitted to, as the cells could be seen becoming more rough around the edges as apposed to the non transfected controls. Ahuja *et al.*, (2005) have described that large T-ag does have the possibility of transforming cells in culture by inhibiting the p53 tumor suppressor gene. However, a study by Djelloul *et al.*, (1997) using Caco-2 cells and SV40 large T-ag

concluded that the large T-ag did not have the possibility of transforming Caco-2 cells in culture. They demonstrated this by showing that the p53 gene in Caco-2 cells is defective, and thus the large T-ag has no binding affinity and therefore cannot commence its oncogenic function.

Immunostaining of the cells transfected with pRc/CMV-SLT had to be done three times since the first two stainings did not show any results. The first substrate used was not strong enough to produce a signal in these cells. The second substrate did not give any results either. The third fluorescent substrate used produced staining in few of the cells in the culture. The reasons the first two substrates did not produce signal could be that they were not suited for this particular cell culture. Another method was used to determine the presence of transcribed large T-ag in the cells, making of cDNA from RNA extracted from the cells. The PCR targeting the large T-ag in the cDNA samples gave a positive result. Caco-2 cells for this particular method had been transfected with DNA 24, and 48 hours to allow for enough time for eventual proteins to be expressed in the cells. The results for expression of the large T-ag protein in this experiment were done as a control to show that the plasmid used in fact is functional. Had there been time in this project, the natural uptake cell cultures would have also been checked for protein expression.

The cell culture samples which had positive results of the PCR targeting the large T-ag did not necessarily indicate that the Caco-2 cells had taken up the DNA. Because there was no time to check whether the DNA actually was taken up in the cells nuclei or associated to the exterior of the nuclei, the PCR detection limit was tested. There were no bands visible below 245 DNA copies, indicating that the PCR was not very sensitive. Several other dilution factors and cycle times could have been checked. This to control more thoroughly how many DNA copies were necessary to get a positive signal, and which was the lowest cycle number needed to produce this signal.

As a conclusion to this study there are several things one could further have explored. The length of exposure time could have included a period of letting the cells grow without any further addition of DNA. And when the period had passed, do the PCR targeting the large T-ag and see if it still would produce a positive signal. The thought is that over time the added DNA would have degraded in the cell, and not been able to be detected by the PCR. The method of harvesting only the cell nuclei (Anker *et al.*, 2004) could also have been

optimized. This because there is a possibility of this method not have functioned to its intent. The process of isolating DNA from the cell nuclei could be a step were association could happen if the DNA added has not passed into the nuclei but is attached to the nucleus membrane. And therefore when isolating DNA from cell cultures, it was not isolated only from the nuclei. But traces of the added DNA could be in other parts of the cell. Also the DNase treatment could better have been optimized. Several more concentrations and time periods of exposure could have been testes to exclude possible DNA association to the nucleus membrane.

When transfected with circular plasmid DNA, the cell cultures when analyzed by immunostaining and by the making of cDNA from RNA harvested from cell cultures gave positive results indicating the presence of DNA inside the cell. If there had been time the same methods should have been applied to the natural uptake experiments. This would have been a better method than the PCR done in this study to determine the presence of DNA within the cell.

The original intent of the study was to use several different cell lines for the experiments. Due to the short time period for this master thesis study, this was not done. As mentioned above a similar study conducted with large T-ag and Caco- 2 cells, showed that the large T-ag does not have the function of transforming this particular cell line (Djelloul *et al.*, 1997). It would therefore have been interesting to see what could have happened in other cell lines.

Lastly more specific analyzing methods could have been used. The PCR results in this study could further have been authenticated by for instance the southern blot hybridization. Or by a more sensitive method like real time PCR.

The objective of this master study was to see if foreign DNA could be spontaneously taken up by mammalian cell cultures. The results of this study can not verify that this is the case. Since the validation of the methods used can't positively confirm the presence of the DNA inside the cell or nuclei.

4. MATERIALS

4.1 Cell line

Caco-2 (ATCC HTB-37) is an immortalized human epithelial cell line with origin from the colon of a 72 year old male Caucasian collected when the individual was diseased with colorectal adenocarcinoma cells. The cells are very similar to non-cancerous epithelial cells between the passages 6-35, before losing the ability to differentiate. The cells grow as adherent culture cells, and when cultivated in lab they require a surface for growth and differentiation. The surface is usually plastic and the bottom is coated with extracellular matrix components to help adhesion.

4.2 Solutions used for cell cultures

Minimum Essential Medium Eagle (MEME) (Sigma-Aldrich, Germany) was prepared by dissolving the content of one glass of produced media into 900 ml of Milli-Q water. The powder was stirred until dissolved. The solution was then added 29.3 ml of sodium bicarbonate solution (7.5% w/v, Sigma-Aldrich, Germany). The desired pH of the solution was 7.0, but the pH was adjusted to 6.8 as the value may rise during filtration. The solution was brought to final volume of 1 L and sterile filtered into an autoclaved bottle using a 0.2 m filter. Before use, a mixture of antibiotics (penicillin-streptomycin-neomycin, Sigma-Aldrich, Germany), MEM non-essential amino acid solution 100x (Sigma-Aldrich, Germany) and Fetal Bovine Serum (Biochrom AG, Germany) were added aseptically to medium. 500 ml of final medium was prepared each time for use, containing 1% antibiotic solution (5 ml), 1 % MEM non-essential amino acid solution 100x (5 ml), 20% Fetal Bovine Serum (FBS) (100 ml) and Minimum Essential Medium Eagle (390 ml).

1 x PBS

Working concentration of 1x was made by diluting Dulbecco's Phosphate buffered saline 10x (Sigma-Aldrich, Germany) with Milli-Q water. PBS was used to wash cells before treating them with trypsin. Some procedures required albumin (from bovine serum), (Sigma-Aldrich, Germany) to be added (PBSA).

Trypsin

Trypsin was used to detach adherent cells from the culture flask. A 0.25% EDTA- Trypsin (Sigma-Aldrich, Germany) solution was used.

4.3 Bacteria

Escherichia coli (*E. coli*) is a common bacteria used in studies as it is easy to grow and genetics are well studied. In this study *E. coli* was used to produce large quantities of plasmid containing the gene construct which is to be exposed to the mammalian cells.

4.4 Growth media used for bacteria

Luria Broth Base (LB) (Invitrogen) was prepared by dissolving 25 g of LB powder in 1 L distilled water and then autoclaved at 121°C for 20 minutes. **LB agar (LBA)** was prepared by adding 12 g agar (Merck) to 1 L of LB and then autoclaved at 121°C for 20 minutes. LB agar was poured into petri-dishes. LB and LB agar were amended with ampicillin to a concentration of 100 µg/ml (LBamp¹⁰⁰).

4.5 Buffers

For purification of plasmids with the QIAGEN plasmid purification kit (Qiagen, Germany), the following buffers were prepared following instructions in the handbook provided by the manufacturer. Buffers prepared contained the ingredients shown in table 1.

Table 1: Buffers used with QIAGEN plasmid purification kit.

Buffer	Composition
P1	50 mM Tris·Cl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A
P2	200 nM NaOH, 1% SDS (w/v)
P3	3 M potassium acetate, pH 5.5
FWB2	1 M Potassium acetate, pH 5.5
QBT	750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v); 0.15 % Triton® X-100 (v/v)
QC	1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)
QF	1.25 M NaCl; 50 mM Tris·Cl, pH 8.5; 15% isopropanol (v/v)

4.6 Green fluorescent protein

Green fluorescent protein (pHrGFP-1, Invitrogen, Germany) is a protein which exudes fluorescent light when viewed under the right lighting conditions. In this study it was used as a positive control for the visualization of DNA uptake (both in natural uptake, and transiently transfected into cells).

4.7 DNA amounts

The amounts of DNA used for the different experiments in the study are listed in the table below.

Table 2: Depicting DNA amounts used and over what time period

	Circular pRc/CMV- SLT	Linear pRc/CMV-SLT	Circular pHrGFP-1
Short term exposure (2 days)	0.5, 1.0, 2.5 & 10, 25 and 50 µg	10, 25 and 50 µg	2.5, 10 and 50 µg
Long term exposure (1 or 2 weeks)	50 µg	50 µg	
Transient transfection	2.5 µg		2.5 µg
DNase treatment	50 µg		

5. METHODS

5.1 Plasmid purification

E. coli cells from glycerol stocks (freeze cultures) containing the pRc/CMV-SLT plasmid were streaked onto an LBamp¹⁰⁰ agar plate and grown overnight in a 37 °C incubator. Stationary phase cultures in LBamp¹⁰⁰ were started by single colonies and incubated overnight with vigorously shaking (200 rpm) at 37 °C. Five flasks were added 500 ml of LBamp¹⁰⁰, and 1 ml of overnight bacterial culture was added into each flask. The flasks were then vigorously shaken at 225 rpm in a 37 °C incubator overnight. The bacterial cells were harvested by centrifugation at 6000 x g for 15 min at 4 °C. The bacterial pellet formed by centrifugation was stored at -20 °C until used. The supernatant was discarded.

Plasmids were purified from stored *E. coli* culture pellets using QIAgen filter plasmid purification kit (Qiagen, Germany) and buffers P1 (resuspension buffer), P2 (lysis buffer), P3 (neutralization buffer), FWB2 (QIA filter wash buffer), QBT (equilibration buffer), QC (wash buffer) and QF (elution buffer). The buffers contained the ingredients as described in table 1 (ref 4.5).

The pellet obtained in the previous step was added 125 ml of buffer P1 and inverted and vortexed until the pellet was dissolved. Solution was then added 125 ml of buffer P2 and inverted before incubated at room temperature for 5 minutes. Buffer P3 was pre-chilled before use and 125 ml was added and the solution inverted to mix before poured onto a filter attached to a collecting bottle. The lysate was then incubated for 10 minutes at room temperature. A vacuum source was connected to the filter and switched on. When all the liquid had passed through the filter, 50ml of buffer FWB2 was added onto the precipitate in the filter and stirred carefully. The vacuum was then switched on until all the liquid had passed through. The QIAgen 10 000 tip was equilibrated with 75ml of buffer QBT before the filtered lysate was applied onto it. The column was let to empty by gravity flow. The column was washed with 600 ml of buffer QC. The plasmid DNA was eluted with 100 ml of buffer QF. The DNA was then precipitated with addition of 70 ml room temperature isopropanol. The solution was mixed and centrifuged at 5000 x g for 60 minutes at 4 °C. Supernatant was carefully decanted and discarded. The pellet obtained by centrifugation was washed with 10 ml 70% room temperature ethanol and centrifuged at 5000 x g for 60 minutes

at 4 °C. Supernatant was carefully decanted and discarded. Pellet was left to air-dry for 10 minutes before re-dissolved in autoclaved water. Concentration of DNA was measured with Nano-drop® ND-1000 (NanoDrop Technologies Inc, USA), before samples were stored at -20°C until used.

5.2 Mammalian cell cultures

Caco-2 cells, passage 22 (p 22) stored in liquid nitrogen (N₂) were thawed and transferred to MEME culture medium containing 20% FBS. Cells were grown and maintained in 20% FBS cell culture medium (MEME) in a 37 °C incubator with 95% air and 5% carbon dioxide (CO₂). Before adding to cell cultures, all solutions were pre warmed to 37 °C. Cells were inspected daily by microscope and when they grew to be 80% confluent they were split in either 1:4 or 1:6, or frozen for storage. After each split, the cell culture was given a new passage number. All solutions were pre warmed to 37 °C before added to cells. All work with cells was conducted in a Laminar Air Flow bench (LAF) under sterile conditions, and using single use plastics (tubes, pipettes, culture flasks) (Nunc, Denmark).

Thawing of cells

Caco-2 cells p 22 were taken from the N₂-tank and thawed in hand. The thawed cell suspension was then transferred into a 15 ml centrifuge tube. Five ml of pre warmed MEME was added drop wise to the cell suspension to prevent the cells suffering osmotic shock. The centrifuge tube was then centrifuged for 3 minutes at 1000 rpm. The supernatant was carefully suctioned off and the cell pellet was re-suspended in 5 ml of MEME. The cell solution was then transferred into a small cell growth flask with a filter cap. Cells were then put into incubator.

Splitting of cells

At 80% confluency, cells were split. Cells were split at 80% confluency (checked in microscope) to prevent them from dying or changing. Before splitting, MEME, 1 x PBS (wash solution) and trypsin (detaches cells from surface) were pre warmed to 37 °C. MEME in cell culture flask was suctioned off and discarded. Cells were then washed once with 1 x PBS to remove all traces of FBS as this inhibits trypsin from working. The 1 x PBS was removed by suction and trypsin was added to cells. The excess trypsin was suctioned off and the flask was put into incubator until cells

detached from the surface (checked by microscope). A fixed volume of fresh MEME was added to detach cells and to stop trypsin from working. Cells were usually split 1:4 or 1:6 and transferred to a new growth flask together with growth medium. 5 ml total for small flasks and 12 ml total for medium flasks. After each splitting, the flask was marked with a new passage number.

Freezing of cells

Cells were washed once with 1 x PBS and detached from flask with trypsin as previously described under splitting. The cell suspension was then transferred into a 15 ml centrifuge tube and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded carefully. The cell pellet was then re-suspended in a 2x freeze solution. The 2x freeze solution was made containing: 50% MEME (without antibiotics and non-essential aminoacids); 40% FBS; and 10% DMSO (Sigma-Aldrich, Germany). 1 ml of the cell and freeze solution was dispensed into 1,8 ml CryoTubesTM (Nunc, Denmark) marked passage number, cell line and date, and frozen over night at -70 °C. The tubes were then transferred into liquid N₂ for storage and backup.

5.3 Natural uptake of DNA

Short term exposure

First day of experiment cells were counted and equivalents of 5×10^6 cells were transferred into each well of a 6 well culture tray (Nunc, Denmark). Second day, DNA was added into the wells. Third day the cells were observed under microscope. Fourth day the nuclei was isolated with cell lysis buffer (10 mM Tris, 10 mM NaCl, 0,2% nonylphenoxypolyetoxy ethanol [NP40], pH 8 (Anker *et al.*, 2000) and DNA was isolated as described in section 5.4.

Long term exposure

Cells were grown in media containing only 1% FBS to keep them from growing to fast/reaching confluency. DNA was added every day for the course of one and two weeks in two different experiments. The last day, nuclei were isolated as described above.

DNase treatment

To check for DNA uptake into cells/nuclei or if it was associated to nuclear membrane, experiments with a very short term exposure was performed. The circular plasmid DNA was only let stay in 6 well trays for seconds before media was suctioned off. DNA was then isolated from cell nuclei and analyzed. To check if DNA was attached to the membrane of cell nuclei, the isolated cell nuclei were DNase treated before DNA isolation and PCR analysis. Two different concentrations, 2 U and 20 U, of DNase I (Roche, Switzerland) dissolved in buffer consisting of HEPES, CaCl₂ and MgCl₂ was used. Cell pellet was dissolved in the DNase solution and incubated at 37 °C for time periods of 15, 30 and 60 minutes. DNA was isolated from cell nuclei and PCR targeting large T-ag was run.

5.4 Genomic DNA isolation

Isolation of DNA was done using the Nucleospin® Tissue XS kit (Macherey-Nagel, Germany). The kit comes supplied with buffers and columns. Protocol for cultured cells was followed. In the pre-lysis step cells were re-suspended in 80 µl of buffer T1 added 8 µl of Proteinase K and incubated at 56 °C in a heating block for 10 minutes. Lysis was done by adding 80 µl buffer B3 and incubated at 70 °C for 5 minutes. Ethanol (96-100%) adjusts DNA binding conditions and 80 µl was added. Solution was then applied into the Nucleospin®) Tissue XS column, and centrifuged at 13000 rpm for 1 minute. Flow through was discarded and 50 µl of wash buffer BE was added in 2 rounds, each following a 1 and 2 minutes centrifuging. DNA was eluted by adding 30 µl of buffer BE and centrifugation for 1 minute. Samples were put in heat block at 90 °C for 3 minutes to remove residual ethanol. DNA concentrations were measured with Nano-drop® ND-1000 (NanoDrop Technologies Inc, USA) before samples were stored at -20 °C until further analysis.

5.5 Polymerase Chain Reaction (PCR)

PCR was run with isolated total DNA samples to first check if the DNA was amplifiable. A human constitutive expressed gene β -actin was used as target. Primer sequence and PCR profile was obtained from the article by Karbownik *et al.*, 2005. To detect target DNA from the plasmid pRC/CMV-SLT added to the cell cultures, primers and PCR programs described in the article by Dhaene *et al.*, 1999 was used. All primers were ordered from Operon (<http://www.operon.com>). Stock solutions of 100 μ M were made by adding dH₂O amount specified by manufacturer. Table 3 shows the primers used in study. For PCR reactions, samples were prepared in 0.2 ml PCR tubes using 2x Dynazyme II mastermix (Finnzymes, Finland) containing DyNAzymeTM DNA polymerase in a total volume of 50 μ l. Table 4 shows the PCR programs used. Actin PCR's were run with MJ Research PTC-200 (Global Medical Instrumentation Inc, USA) thermocycler, and PCR targeting large T-ag were run with GeneAmp® PCR system 2700 (Applied Biosystems, USA) thermocycler.

Table 3: Primers used in this study

Name	Sequence 5'-3'	References
β -actin antisense	5'-ACT CCA TGC CCA GGA AGG A-3	Karbownik <i>et al.</i> , 2004
β -actin sense	5'-TGA CCC AGA TCA TGT TTG AGA-3'	Karbownik <i>et al.</i> , 2004
SLT sv. for3	5' -TGA GGC TACT GC TGA CTC TCA ACA-3'	Dhaene <i>et al.</i> , 1999
SLT sv. rev	5' -GCA TGA CTC AAA AAA CTT AGC AAT TCT G -3'	Dhaene <i>et al.</i> , 1999

Table 4: PCR programs used in study

Name	Program	Amplification fragment size(bp)
Actin	1) 95°C 5 min 2) 30 cycles of: 95°C 1 min 60°C 1 min 72°C 1 min 3) 72°C 10 min	484
Large T-ag	1) 94°C 5 min 2) 35 cycles of: 94°C 1 min 63°C 1 min 72°C 1 min 3) 72°C 5 min	105

Gel electrophoresis

PCR products were analyzed by loading them on an agarose gel added Ethidium Bromide (EtBr) (Sigma-Aldrich, Germany). PCR products targeting actin were separated on a 1% agarose gel made by mixing 0.8 g agarose with 80 ml 1 x TAE (Tris-acetate-EDTA) electrophoresis buffer. PCR products targeting large T-ag were separated on a 2% agarose gel made by adding 1,6 g agarose in 80 ml 1 x TAE buffer. The solutions were heated in a microwave until agarose had melted, and before 8 µl of EtBr was added. The solution was cooled to approximately 65 °C before it was poured into a 20 x 15 cm electrophoresis chamber where it was left to polymerize for 30 minutes. 10 µl of PCR product was mixed with 2 µl of loading buffer (6xT) and loaded into wells. A ladder with fragments of known sizes was loaded alongside samples for size determination. For actin samples, 5 µl 1 kb plus ladder (Invitrogen, Germany) was used. And for large T-ag PCR runs 5 µl of 50 bp Step ladder (Sigma-Aldrich, Germany) was used. The gels were viewed under UV-light as the EtBr intercalates the nucleotides in DNA and therefore makes it visible.

5.6 Transient transfection of mammalian cells

In control experiments transient transfection with pRc/CMV-SLT and pRcGFP-1 was performed using LipofectamineTM (Invitrogen, Germany) according to instructions from the manufacturer regarding number of cells and amount of DNA. Cells were split and let grow in either 6 well tray or small cell culture flask for one day before they were transfected. The third, day cells were observed in microscope. Then the fourth day nuclei and DNA was isolated as described in section 5.3 and 5.4.

5.7 Immunoperoxidase staining of cells

Cells transfected with plasmid DNA of pRc/CMV-SLT were stained with two different substrates to determine if the large T-ag was expressed.

Cells were grown in a 6 well tray and transfected as described above with plasmid DNA of pRc/CMV-SLT. Fourth day, medium was removed and cells were washed with 1 x PBS before they were fixated with methanol at room temperature for 10 minutes. Cells were then washed with 1 x PBS before 1 x PBSA was added. Cells were then incubated for 10 minutes at 37 °C. 1 x PBSA was removed and 1 ml of primary antibody (Anti-SV40 T Antigen [Ab-2] Mouse mAb, Calbiochem, USA) diluted 1:100 from stock solution in 1 x PBSA, was added and cells were incubated at 37 °C for 45 minutes with shaking every 15 minutes. Cells were washed with 1 x PBS before 1 ml of secondary antibody (Polyconal Rabbit anti mouse Immunoglobulins, Dako, Finland) diluted 1:200 from stock solution in 1 x PBSA was added. Cells were incubated for 30 minutes at 37 °C with shaking every 10 minutes. Cells were washed with PBS before 1 ml of SigmafastTM OPD (Sigma, Germany) substrate was added. Cells were kept in dark until staining was observed. The reaction was stopped by adding 1-2 ml of dH₂O. Same procedure was done twice because the first substrate was too weak. So for the second time a different substrate was used. Substrate was Immunopure® Metal Enhanced DAB (Pierce, USA), 600 µl was diluted in 5,4 ml of Stable peroxide buffer (Pierce, USA). Substrate was left on until staining observed, and reaction was then stopped with dH₂O.

5.8 Immunofluorescence detection of large T-ag

Cells were grown in two different 8-chamber slides and transfected with plasmid DNA of pRc/CMV-SLT for 24 and 36 hours. After this, cells were fixed by adding 150 µl of 4% paraformaldehyde in 1 x PBS into each chamber and incubating at room temperature for 30 minutes. Cells were then washed with 1 x PBS before permeabilized with 1 x PBS with 0,5% Triton X-100 for 15 minutes at room temperature. Primary antibody (Anti-SV40 T Antigen [Ab-2] Mouse mAb Calbiochem, USA) diluted 1:100 in PBSA was added, and the cells were stored at 4 °C overnight. Cells were then washed with 1 x PBS before incubated for 1 hour with fluorescent substrate; AlexaFluor 488; goat anti-mouse IgG F(ab')₂ (Invitrogen, Germany). Top of the slides was then removed and bottom was washed with 1 x PBS three times and air dried before 1 drop of Vectashield Mounting Medium (Vector Laboratories, USA) was added onto each frame. Cover slip was mounted and plates stored at room temperature in the dark before observed under a fluorescent microscope.

5.9 RNA isolation, cDNA synthesis and PCR to detect transcript of large T-ag

Three small cell culture flasks were grown with Caco-2 cells and two of them were transfected with circular plasmid DNA of pRc/CMV-SLT for 24 and 48 hours. Third flask was used as control with no DNA added. Cells were then spun down to pellet and supernatant discarded. Cell pellets were then used to extract RNA by using QIAgen AllPrep DNA/RNA/Protein kit (QIAgen, Germany). Concentrations of the RNA samples were determined with Nano-drop® ND-1000 (NanoDrop Technologies Inc, USA). cDNA was made by using a Superscript™ III Reverse-Transcriptase kit by Invitrogen (Germany). The cDNA was used as template for the large T-ag PCR.

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