Mis-splicing of the insulin gene in BK-virus infected cells - Dependent on virus transformation?

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RÉSUMÉ

The polyoma-BK-virus-transformed rat pancreatic islet cell line (6A3) expresses viral T-antigen, has highly malignant characteristics and has lost insulin-secreting capacity. In 6A3 cells, the phosphoinositide hydrolysis is constitutively activated, with a substantial loss of regulatory control. The changes in the trans-signal system appear to be located at the level of phospholipase C and its GTP-binding protein. Recent studies have shown that 6A3 cells are derived from insulin-producing β-cells of the pancreas. The absence of insulin production is most likely manifested in the mis-splicing of the insulin gene by retention of intron A. Therefore, the working hypothesis of this study was to investigate if mis-splicing of the insulin gene in 6A3 cells is triggered by BK virus transformation. For that purpose we used a two non-insulin producing cell model systems, hamster kidney cells that are transfected with BK virus (BKT-1B) and the corresponding non-transformed cell line (BHK-21) as a control. A plasmid vector that contained the entire genomic DNA of human insulin (pcDNA3.1_ins) was constructed and afterwards introduced to the cells by lipofectamine method. By this approach it would be possible to monitor if the insulin gene can be properly spliced in the BK-transformed cells compared to non-transformed cells. The results showed the mis-splicing of the human insulin gene is not affected by BK-transformation as judged by RT-PCR and gelelectrophoresis.

1. INTRODUCTION

The human polyomavirus BK was first isolated in 1971 from the urine of a renal transplanted patient [1,2]. Primary infection occurs early in childhood, and usually establishes itself in a latent state in the kidney [2,3,4]. BK virus persistently infect 75-80% of the human population [2,3,4]. Initial infections rarely cause clinical disease, although respiratory symptoms and urinary tract disease do occur [5]. Little is known about transmission route, but oral or respiratory route is indicated by induction of upper respiratory disease by BK virus and detection of latent BK virus DNA in tonsils [6]. Reactivation from latent state may occur during immunosuppressive disease or treatment and during pregnancy [7,8]. Active infection has been associated with hemorrhagic and non-hemorrhagic cystitis, uretic stenosis, tubulointerstitial nephritis, asymptomatic nephritis, interstitial desquamative pneumonitis, atypical retinitis, meningoencephalitis, hepatic dysfunction, and autoimmune diseases [7,8]. Polyomavirus-induced nephropathy, caused by BK virus, has emerged as a significant cause of severe renal allograft dysfunction and ultimate graft loss [4]. BK virus has a proven potential for malignant transformation of numerous cell cultures [2], but there is no conclusive proof that BK virus directly cause or act as cofactor in human cancer [4,5]. Human cancers reported to be associated with BK virus are; glial tumors, insulinoma, osteosarcoma and colorectal tumor among others [4]. Adenocarsinomas of pancreas are commonly seen following rodent BK virus inoculation [7], and BK virus has been identified in human insulinomas [9].

Transformation of rat pancreatic islet-cell lines by BK virus (TU strain) infection *in vitro,* has been shown to affect the insulin secretion [2]. The BK virus-transformed islet cells continued to secrete decreasing amounts of insulin and lost their insulin-secreting capacity seven weeks after transformation. The reason for this event is not clear. In SV40-transformed rat pancreatic islet cells it has been detected a 30-kDa protein immunologically related to insulin, but the size does not match that of a pre-pro-insulin mRNA [10]. Other reports of transformed pancreatic islet cells have indicated insulin levels below 1 % of that of native β-cells [11].

Unpublished data have shown rat pancreatic islet-cell lines transformed by BK virus (6A3 and 5A4 cells) to mis-splice the insulin gene by retention of intron A (figure1). The absence of insulin production and secretion is most likely dependent on this event. Therefore, the working hypothesis is that mis-splicing of the insulin gene in BK virus infected cells is

dependent on virus transformation, resulting in a non-functional insulin precursor not able of being processed. The purpose of this study is to test whether this hypothesis is correct. For this purpose, we cloned the human insulin gene into a plasmid vector (pcDNA3.1) capable of expressing the insulin gene. The plasmid was denominated pcDNA3.1_ins. As a cell model systems we chose baby hamster kidney cell lines, BHK-21 cells which are non-transformed (and served as control in the experiments) and BKT-1B cells which were transformed with the BK virus. The cells were transfected with pcDNA3.1_ins and transient expression of insulin was judged by gel electrophoresis and RT-PCR..

2. MATERIALS AND METHODS

2.1 Materials

1Kb Plus DNA ladder, agarose, TOPO TA Cloning Kit, the plasmid vector pcDNA3.1(+), BHK-21 cells, Lipofectamine Reagent, G418 and Plus Reagent were purchased from Invitrogen Corporation; primers were synthesized by Sigma-Aldrich; Jumpstart REDTaq ReadyMix was purchased from Sigma-Aldrich; Trizol was from GIBCO BRL; Ready-To-Go RT-PCR Beads was purchased from Pharmacia Biotech; PCR Markers was purchased from Promega Corporation; T4 DNA Ligase and restriction enzymes BamH1 and EcoR1 were purchased from Takara Bio inc; PCR-machines used were PTC-200 (Peltier Thermal Cycler), MJ Reasearch, and PTC-100 Programmable Thermal Cycler, Hybaid; Minimum Essential Medium was purchased from ATCC. All other chemicals used in this stydy were of standard laboratory grade.

2.2 RNA preparations

Total RNA was isolated directly from cell samples with TriZOL™ and according to the manufacturer's protocol. After removal of the cell culture medium, 750 µL TriZOL™ reagent was added to each cell container to achieve lysis of the cells. The cells were detached from the container by a cell scraper and homogenized before they were placed in a sterile test tube. The sample suspension was incubated in room temperature for 5 min to permit complete dissociation of the nucleoprotein. To separate the aqueous phase containing RNA, 200µl

chloroform was added and the mixture was incubated for 10 min at room temperature and than centrifuged 15 min at 12,000 x *g* at 2 to 8°C. The aqueous phase was than transferred to a new sterile test tube and 500 µl of isopropanol was added to precipitate RNA. After 10 min incubation at room temperature and centrifugation for 10 min at 12,000 x *g* at 2 - 8°C, the supernatant was removed. The RNA pellet was washed once with 1.0 ml 75 % ethanol. After centrifugation at 7,500 x *g*, the RNA was re-dissolved in 50 µl of RNase-free water. The sample was then incubated 10 min at 59°C in a water-bath to dissolve the RNA completely. Quantity and quality of total RNA was judged by spectrophotometry at a wavelength of 260 and 280 nm, respectively.

2.3 Gel electrophoresis

PCR products and restriction enzyme products were separated by electrophoresis at 110 V for approximately 1 hour in 1xTBE buffer, using 0.8 % to 1,5 % agarose gels, depending on the size of DNA fragments. Products were visualized by staining with ethidium bromide. Pictures of gels with products were taken under UV-light exposing. The molecular weight marker (1Kb Plus DNA ladder, Invitrogen) was used to estimate the base pair length of products separated.

2.4 Cell culture and transfection

BHK-21 cells [ATCC CCL 10] and BKT-1B cells [12] were cultured in RPMI-1640 (11mmol glukose) medium supplemented with 10% FBS (fetal bovine serum), 75 µg/ml penicillin and 50 µg/ml streptomycin and incubated in a humidified atmosphere with 95 % air and 5% CO_2. Cells were tryptinized and sub-cloned once or twice weekly. The medium was changed twice weekly and 24 hours prior to an experiment. The experiments were always performed with cells in the logarithmic phase of growth.

BHK-21 cells and BKT-1B cells were transfected with pcDNA3.1_ins using Lipofectamine Reagent and Plus Reagent (Invitrogen) according to manufacturer's protocol. A 6 well cell culture plate with approximately 60 % confluent cells was prepared prior to transfections. Approximately 1 µg pcDNA3.1_ins was used for each transfection. The growth medium was replaced with medium containing G418 (400µg/ml) for selection of

pcDNA3.1_ins transformed cells. Cells were usually harvested between 24 to 72 hours after transfection by scraping in Trizol for later RNA preparations.

2.5 Polymerase chain reaction (PCR)

PCR was preformed using Jumpstart REDTaq ReadyMix (Sigma) and carried out according to manufacturer's protocol. PCR reactions contained 12,5 µl Jumpstart REDTaq ReadyMix, 1 µl forward primer, 1 µl reverse primer, 4,5 µl water and 1 µl DNA template solution. Specifications of primers used are listed in table 1. Primers used in PCR amplification of the human insulin gene, inserted nucleotide sequences for the restriction enzyme EcoR1 at the 3' end of the fragments and BamH1 at the end 5' of the fragments. The efficiency and specificity of the primers was tested using a temperature gradient form 55.5°C to 69,8°C to find the optimal PCR condition for amplification of the human insulin gene from human genomic DNA. Cycles were carried out at 94°C for 1,5 min, respective temperature °C for 30 sec and 72°C (30 cycles) with a final elongation step at 72°C for 5 min.

PCR reactions for the rat insulin II gene were carried out with the following corresponding primers. Forward primer 5'AGCCCTAAGTGACCAGCTACAGTC3' and reverse primer 5'TTTATTCATTGCAGAGGGGTGGAC3'. The predicted product sizes of the rat insulin II primers are 423 bp and 1047 bp for mRNA and genomic DNA, respectively as previously described [13].

Primers for amplification of insulin fragments were designed using sequence information for the human insulin gene (Accession no. NT_009237) and checked for cross-activity and specificity using BLAST. For amplification of insulin gene from human genomic DNA the following primers have been designed, including restriction sites for BamH1 and EcoR1: Forward primer: 5'-CCT-GCC-TGT-CTC-CCG-GAT-CCC-TGT-CCT-TCT-GC-3' and reverse primer 5'-GGT-GTG-GGG-CTG-AAT-TCG-GGC-TGC-GTC-TAG-TTG-3'. For verification of splicing products in transfected BHK_21 and BKT-1B cells the following primers were used: Forward primer: 5'-ATC-AGA-AGA-GGC-CAT-CAA-GC-3' and reverse primer: 5'-GCT-GGT-AGA-GGG-AGC-AGA-TG-3'. The amplified PCR products were verified by DNA sequencing with the ABI 3130 XL instrument.

2.6 Plasmid construction

pcDNA3.1(+) contains a CMV promoter, multiple cloning site, a neomycin resistance gene and a ampicillin resistance gene. The multiple cloning site contains nucleotide sequences

for the restriction enzymes BamH1 and EcoR1. Ampicillin resistance was used for selection of pcDNA3.1(+) transformed bacteria. The multiple cloning site was used for unidirectional insertion of DNA fragments into the plasmid vector.

TOPO TA Cloning Kit contains the vector pCA®II-TOPO cleaved by Topoisomerase1, making it unnecessary to use DNA ligase [14]. Topoisomerase1 breaks a phosphodiester bond in the vectors phosphodiester backbone. The energy from breaking the bond is conserved in Topoisomerase 1's own phosphor-tyrosyl bond to the vector. This energy is used to ligate a DNA fragment into the vector without the need of DNA ligase. PCR products produced using Taq polymerase and plasmids cleaved by Topoisomerase1 have complementary overhanging, single deoxy-nucleotides. This ensures efficiently ligation. pCA®II-TOPO include a ampicillin resistance gene, used for selection of transformed bacteria.

PCR amplified insulin fragments were ligated with pCA®II-TOPO according to manufacturer's protocol. DH 5α bacteria (TOP cells included in the kit) were transformed with pCA®II-TOPO_ins to clone composed plasmid. Insulin fragments were cleaved by restriction enzymes BamH1 and EcoR1 according to manufacturer's protocol. The digested insulin fragments were then ligated into the pcDNA3.1(+) vector using T4 DNA ligase according to manufacturer's protocol.

2.7 Transformation of bacteria with plasmid vectors

50 µl frozen, competent, E.coli cells (DH 5α) were mixed with plasmids (pcDNA3.1 or pCR2.1–TOPO) and incubated for 30 min at ice. Cells were then heat-shocked at 42°C for 30 sec without shaking and placed on ice. 250 µl S.O.C. medium of room temperature was added, before the solution was incubated for 1 hour in a shaking incubator at 37°C and 200 rpm. 50µl-100µl of resulting culture in were spread on pre-warmed LB agar plate containing ampicillin and incubated overnight. Single colonies were transferred to separate tubes with 5 ml LB medium containing ampicillin and incubated overnight in a shaking incubator at 37°C with 220 rpm.

2.8 Plasmid preparations

Plasmids were isolated using the QIAprep®Spin Miniprep Kit (Qiagen) and according to the manufacturer's protocol. The Quality and quantity of the plasmid preparations was estimated by spectrophotometry. The typical yield of purified plasmid DNA from 1 ml culture medium was 4,5 μg. The plasmid DNA was reconstituted in water and were used directly in transfection experiments into BHK-21 and BKT-1B cells without further purification.

3. RESULTS

Rat pancreatic islet-cell lines infected by BK virus (6A3 and 5A4 cells) mis-splice insulin with retention of intron A (figure 1). The retention of intron A was verified by DNA sequencing. Data was based on extracted total RNA from Rin5F rat cells (ATCC no.:CRL-2058), rat islet of Langerhans and BK virus transformed 6A3 and 5A4 rat cells [2]. mRNA was transcribed and the resulting cDNA was fractionated by electrophoresis. Figure 1 displays differences in base pair length of cDNA fragments coding for insulin from the respective cells. The resulting fragments were sequenced, and found to be insulin, but with retention of intron A in 6A3 and 5A4 cells.

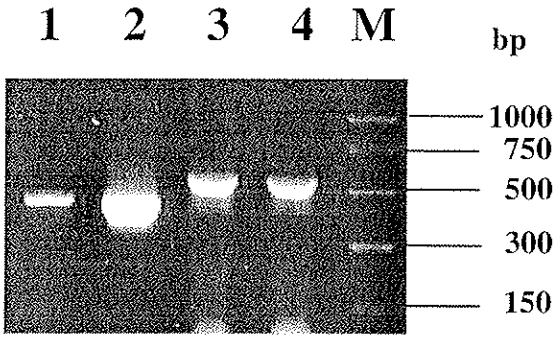

Figure.1. Amplification of rat insulin II in rat pancreatic cells and islets of Langerhans. Insulin was amplified by RT-PCR. The amplified products were fractionated on a 1.5% agarose gel and subsequently stained with ethidium bromide as described in detail in Material and Methods. Lanes: M, 100 bp DNA molecular weight standard (GIBCO BRL); 1, Rin5F cells; 2, islets of Langerhans; 3, BK virus -transformed pancreatic cell line 6A3; 4, BK virus -transformed cell line 5A4. Amplified products were verified by sequencing. Products amplified in Rin5F and islets of Langerhans show the expected molecular weight of 423 bp. Amplification of insulin in BK virus -transformed cells resulted in larger product of 541 bp due to mis-splicing of the insulin gene, including the sequence from intron A of the rat insulin gene which consists of 118 bp.

To test the hypothesis that BK virus.transformation is responsible for mis-splicing, the human insulin gene was cloned into a plasmid vector, pcDNA3.1, that is capable of expressing the insulin gene in eukaryotic cells. For this purpose, human genomic DNA was used to amplify the insulin gene. The optimal amplification conditions of the insulin gene was tested by running a temperature gradient of PCR reactions, as described in Materials & Methods. The expected molecular size (in bp) of the PCR product was 1183 bp. The quality PCR product amplification was judged by gel electrophoresis. The annealing temperature of 63,4°C gave the best result and amplified insulin fragments were finally verified by DNA sequencing. The tested plasmids were denominated pcDNA3.1_ins.

The amplified PCR fragments contained restriction sites for the restriction enzymes BamH1 and EcoR1 introduced by specifically designed primers as described in Materials and Methods. The amplified insulin fragment was than inserted into pcDNA3.1(+) expression vector by molecular cloning as outlined in figure 2A. The ligation of insulin fragment into the vector was unsuccessful as judged by gel electrophoresis and sequencing. Therefore, a second cloning strategy was used, ligating insulin fragments into the pCA®II-TOPO vector to facilitate the digestion by restriction enzymes (see figure 2B). Successful ligation was confirmed by gel electrophoresis and sequencing (figure 3). The digested insulin fragment was purified and ligated into the pcDNA3.1 vector by uni-directional cloning. Restriction analysis and gel elctrophoresis confirmed successful insertion of the insulin gene into the pcDNA3.1(+) vector. The resulting vector was denominated pcDNA3.1_ins and was used for transformation into BHK-21 and BKT-1B cells for expression of the human insulin gene as described below.

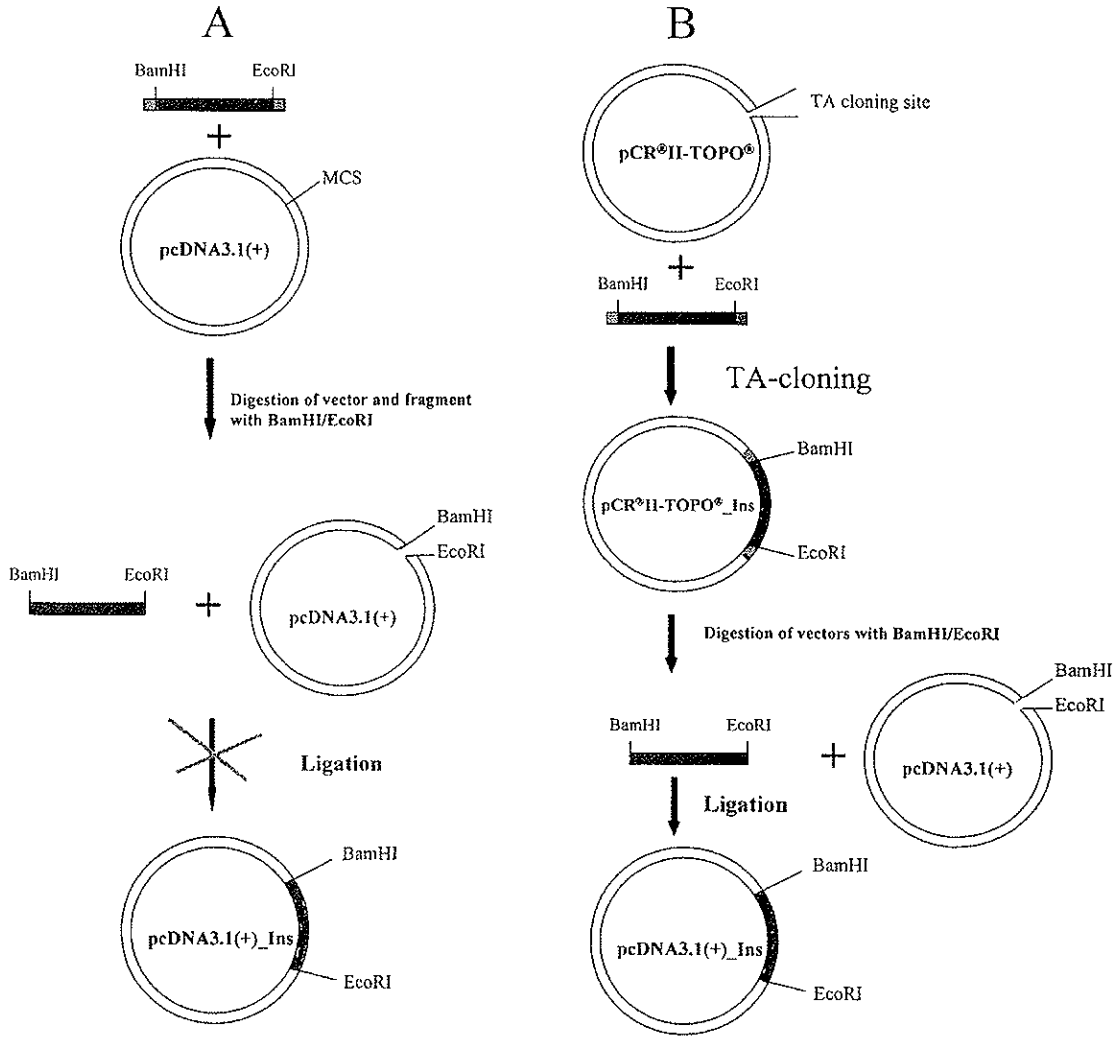

Figure 2: Cloning strategies constructing the pcDNA3.1_ins transfection plasmid. *Strategy A)* The insulin gene and the pcDNA3.1(+) vector were separately digested with EcoR1 and BamH1 before ligation. Successful digestion should give the products complementary sticky ends, making it possible to ligate the gene unidirectionally into the vector, but ligation was unsuccessful. *Strategy B)* PCR amplified, undigested insulin gene was ligated into the pCA®II-TOPO vector to facilitate the digestion of the insulin gene by the restriction enzymes. The pcDNA3.1(+) vector and the recombinant pCA®II-TOPO- insulin plasmid was separately digested by restriction enzymes, before the insulin gene was uni-directionally cloned into the pcDNA3.1(+) vector.

After the bacterial transformation, plasmid DNA was isolated as described in Materials & Methods. The purified plasmids were digested with the restriction endonucleases Bam H1 and EcoRI and further subjected to gel electrophoresis for verification. The resulting fragment had the expected size of 1183 bp. The fragments were verified by sequencing before amplification and purification of pcDNA3.1_ins plasmid for transfection experiments.

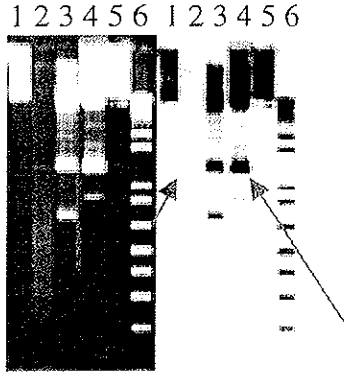

Figure 3: Verification of the insulin expressing vector pcDNA3.1_ins.
Restriction analysis of plasmid vectors separated by gel electrophoresis as described in Materials & Methods (same gel shown twice but with different photographical properties). Lanes: 1, Undigested pCR2.1-insulin; 2, insulin fragment amplified by PCR (short arrow, 1183 bp); 3, BamH1 and EcoRI digested pCR2.1_ins; 4, BamH1 and EcoRI digested pcDNA3.1_ins (insulin fragment, indicated by long arrow, 1183 bp); 5, undigested pcDNA3.1-insulin; molecular weight marker (1Kb Plus DNA Ladder, Invitrogen).

BHK-21 and BKT-1B cells were transfected with the pcDNA3.1_ins plasmids as described in Materials and Methods. One 6 well plate was prepared as outlined in figure 4 below. 24 hours after transfection growth medium was replaced with medium containing G418 (400μg/ml) for selection of transformed cells. Control cells, not transfected with pcDNA3.1_ins were monitored for occurring cell death. Cell death was confirmed by microscopy 24 hours after G418 exposure.

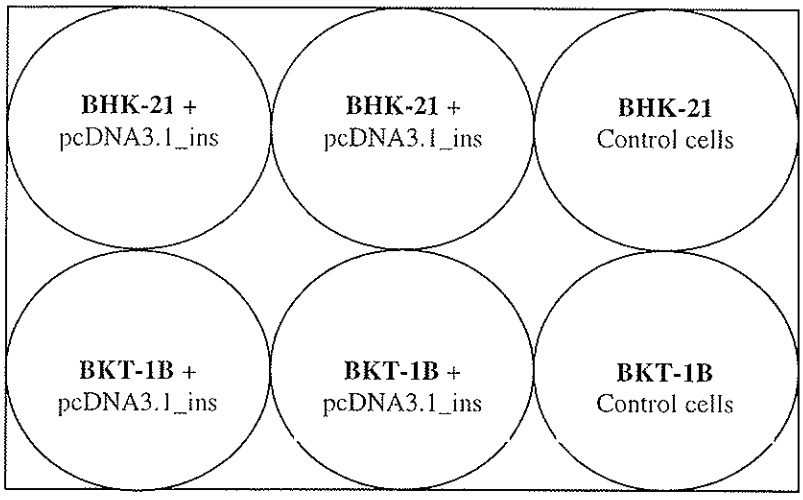

Figure 4: Configuration of the 6 well plate containing cells involved in transfection with pcDNA3.1_ins. BKT-1B and BHK-21 cells were transfected with the pcDNA3.1_ins with the Lipofectamine method as described in Materials & Mehtods. Transfections were confirmed by exposing cells with Geneticin (G418) and by monitoring cell death of the non-transfected control cells.

72 hours after G418 treatment total RNA was isolated from transfected BHK-21 and BKT-1B cells with TriZol (GIBCO BRL), according to manufacturer's protocol. Totoal RNA was converted to cDNA with Ready-To-Go RT-PCR Beads (Pharmacia Biotech), according to manufacturer's protocol. cDNA representing insulin mRNA was then amplified with Ready-To-Go RT-PCR beads (Pharmacia Biotech) and finally subjected to gelelectrophoresis. The result of the experiment is depicted in figure 5.

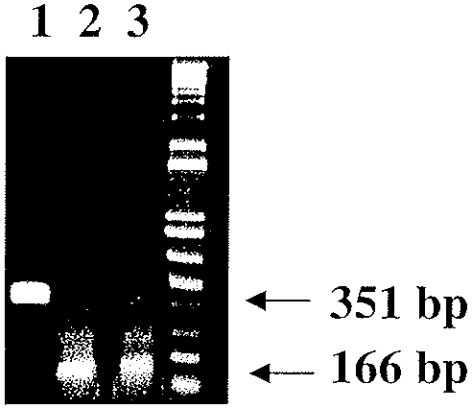

Figure 5: Amplification if insulin fragments by RT-PCR.
Insulin was amplified by RT-PCR as described in Materials & Methods. The amplified products were separated on a 1.5 % agarose gel and subsequently stained with ethidium bromide. Lanes: 1, genomic human DNA (control); 2: insulin fragment in BHK-21 cells tasnfected with pcDNA3.1_ins; Lane 3: insulin fragment BKT-21 cells transfected with pcDNA3.1_ins; M: 1 Kb Molecular weight standard (Invitrogen).

Results show amplification of a 166 bp fragment in both cell lines tested. As control served human genomic DNA. Amplification resulted in a fragment with the expected molecular size of 351 bp (see figure 5).

4. Discussion

Recently, Haukland et al. (ref.no.) established BK virus infected rat pancreatic islet-cell lines, 6A3 and 5A4 cells that have lost the ability to produce and secrete insulin [2]. In addition, mis-splicing of the insulin gene with retention of intron A has been observed in these cells (unpublished data and figure 1). BK virus encodes six viral proteins: large tumor antigen, small tumor antigen, an agnoprotein and three capsid proteins [6]. Studies have suggested a regulatory role for agnoproteins at the levels of viral transcription, replication, capsid protein translation, and assembly [8]. Agnoprotein is also shown to dysregulate the cell cycle by altering the expression of several cyclins and their associated kinases [8]. Large tumor antigen is required for the transforming activities [3]. Tumor antigens has been shown to induce DNA damage, inhibit p53 response to DNA damage and induce cell cycle progression in the absence of mitogenic signals [3,5]. Knowing this, perhaps the most likely mechanism by which BK virus could contribute to mis-splicing of the insulin mRNA is through the mutagenic activity of large T antigen. Splicing reactions must occur with exquisite precision to ensure that a functional RNA molecule is formed [15]. Only a single base change in DNA at splice junction is needed for mis-splicing to occur, leading to nonfunctional mRNAs – as is the case of β-thalassemia [15]. The agnoprotein regulating viral transcription may theoretically also interfere with host DNA transcription. These are just two of many possibilities, and understanding the pathogenesis will need further investigation.

Therefore, the hypothesis of this study was to further investigate if BK virus-transformation is the cause of mis-splicing. For this purpose, a plasmid vector was constructed, containing genomic sequence for human insulin. This vector was than introduced into BK virus-transformed baby hamster kidney cells, BKT-1B cells. As control served non-transformed cells, BHK-21 cells. A non-pancreatic cell model was chosen for the experiments to ensure that the eventual effects on splicing are solely due to BK virus-transformation.

The human insulin gene was introduced by molecular cloning techniques into the plasmid vector, pcDNA3.1.This vector contains a CMV promoter that enables inserted DNA to be expressed in mammalian cells [14], an ampicillin resistance gene for selection of

transformed bacteria and a neomycin resistance gene for selection of insulin transcribing mammalian cells. The restriction enzyme sites BamH1 and EcoR1 in the multiple cloning site were used for unidirectional insertion of the insulin gene into the vector.

To transfer the human insulin gene into the pcDNA3.1(+) vector, two different strategies had to be tried, as shown in figure 4. It was not possible to clone the insulin gene directly into an expression vector as outlined in figure 2A. Digestion of the insulin fragment most possibly failed due to the small size of the fragment. Therefore, TOP TA cloning was used which provided a highly efficient one-step cloning strategy for the direct insertion of Taq polymerase-amplified PCR products into a plasmid vector. Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearized vector has a single, overhanging 3' deoxythymidine (T) residue. This allows PCR inserts to ligate efficiently with the vector. This method has been shown to work as outlined in figure 2B. After introduction of the insulin fragment into the pCr®II-TOPO vector, the insert was possible to digest with Bam H1 and EcoR1. This fragment was than successfully cloned into the expression vector pcDNA3.1 mammalian expression vector using the respective restriction sites for uni-directional cloning.

After transfection of BKT-21 cells with this vector, splicing of the insulin gene was analyzed by RT-PCR. The results showed that the insulin gene was properly spliced in BK virus transformed cells, BKT-1B, and the non-transformed, BHK-21 cells. In addition, amplification of the insulin fragment from human genomic DNA confirmed primer specificity by amplifying the correct product.

In conclusion, the results shown in this study indicate that BK virus-transformation is not the cause of mis-splicing of the human insulin gene, which means that further studies need to be done to further clarify mis-splicing of insulin in BK virus-transformed pancreatic islet 6A3 cells.

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