The Role of Amino Acid 128 in the Induction of Chromosomal Aberrations by the SV40 Large T antigen

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ABSTRACT

Simian Virus 40 (SV40) is a monkey polyomavirus, which causes a lytic infection in simian host cells. When human cells are infected with the virus, many exhibit chromosomal damage and some become immortal (Chang et. al., 1997). In 1990, Ray et. al. reported that the SV40 early gene, Large T antigen (Tag), alone is necessary and sufficient to cause the observed cancer-like chromosomal damage within 48 hours of transfection. Since Tag is a nuclear protein with the ability to bind DNA, it was assumed that Tag must bind the DNA in order to cause chromosomal damage. This was supported by the research of Ray et. al. in 1998, when they created a mutation in the nuclear localization signal of Tag, which changes a lysine to an isoleucine. This mutation produces a cytoplasmic protein, which is unable to damage DNA. Other research has shown that if that same lysine is changed to a threonine, the protein is cytoplasmic, but retains the ability to cause chromosome damage at the magnitude of the wild-type protein (Woods et. al., 1994). Unfortunately, a confounding problem was that the plasmid constructs used for each of these experiments were different. The goal of the current research is to insert the wild-type Tag and the two mutant Tag genes into identical plasmid constructs and ascertain the effect of each on human fibroblast cells. Currently, the wild type and the lysine to isoleucine mutant genes have been successfully inserted into the pIND inducible plasmid. Future work will include obtaining pIND plasmid containing the lysine to threonine mutation of the Tag gene, as well as tissue culture assays to determine the effects of each of these genes in human fibroblast cells.

INTRODUCTION

Simian Virus 40 (SV40) is a monkey polyomavirus. In Green Monkey kidney cells (CV-1) the virus stimulates successive rounds of DNA synthesis without mitosis, this has been termed the greater than G2 phase (>G2). *In vitro* infection of CV-1 cells with SV40 causes more than 80% of the population to enter a >G2 cell cycle phase within two days. It has been suggested that SV40 has evolved a way to interfere with the normal pathway for activating M-phase-promoting factor (MPF), which is necessary for entering mitosis (Scarano et. al, 1994). The mechanism for this is still being investigated.

"Carcinogenesis is a multifactoral process and a large body of evidence indicates that chromosomal aberrations and karyotypic instability plays a causal role in the development and progression of tumors in man" (Stewart and Bacchetti, 1991). In 1962, Eddy et. al. injected the SV40 virus into lab animals and observed tumorogenesis, and since this ground breaking experiment the SV40 virus has been studied in depth (Gersh and Ray, 2002). When human cells are infected with the virus, many cells exhibit chromosome damage and some become immortal (Chang et. al., 1997). In 1990 Ray et. al. reported that the SV40 early gene, Large T antigen, alone is necessary and sufficient to cause the observed chromosomal damage within 48 hours of infection. Damage is seen as both structural (Fig. 1) and numerical (Fig. 2) chromosomal abnormalities. T-antigen can also indirectly cause anchorage independence, loss of contact inhibition, colony formation in low serum, immortalization, and tumorigenesis, all of which are required for the formation of neoplastic cells (Ray et. al., 1990). Although T-antigen has been studied as a model for human cancers since the 1970s, the mechanism by which it causes cancer is still unknown and many possibilities have been identified (Chang et. al., 1997).

T-antigen is one of the first genes transcribed once the virus has entered the host cell. It is a nuclear protein thought to help the virus stimulate the cells to enter the cell cycle. The T-antigen protein contains many functional domains including, ATPase, Helicase, Nuclear Localization Signal, p53, Rb, p300, p107, and DNA binding regions (Fig. 3). Previous research has shown that a point mutation at amino acid position 128 interrupts the nuclear localization signal and causes the protein to be cytoplasmic as opposed to nuclear (Fig. 4). Ray et. al. found that changing the lysine at position 128 to an isoleucine (K128I) interrupts the nuclear localization signal (NLS) and results in no significant chromosomal damage (1998). This result was expected based on the assumption that the protein must have contact with the DNA in order to cause chromosomal damage (Ray et. al., 1998).

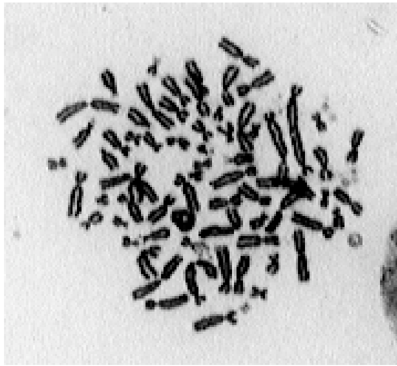

Fig. 1 Structural Chromosome Damage induced by T antigen (Photo courtesy of Dr. F.A. Ray)

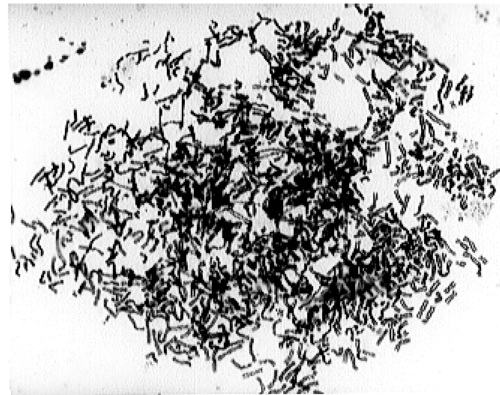

Fig. 2 Numerical Chromosome Damage induced by T antigen (Photo courtesy of Dr. F.A. Ray)

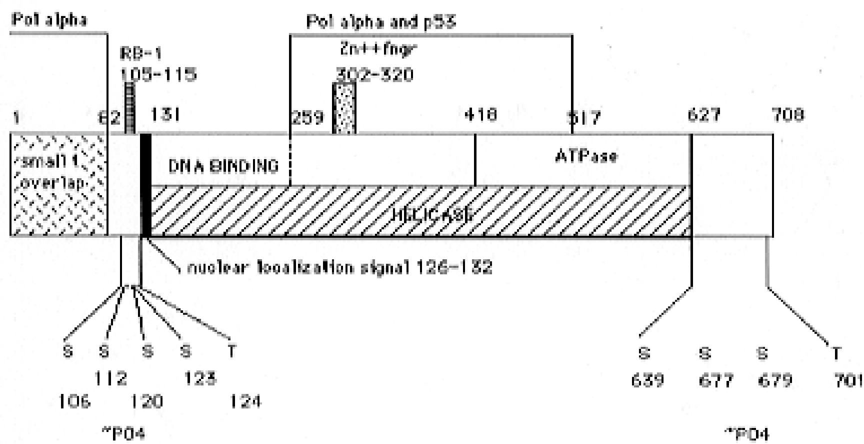

Fig. 3

Current map of functional domains of the SV40 T antigen. Modified from Fanning and Knippers, Annual Review of Biochemistry, 1992.

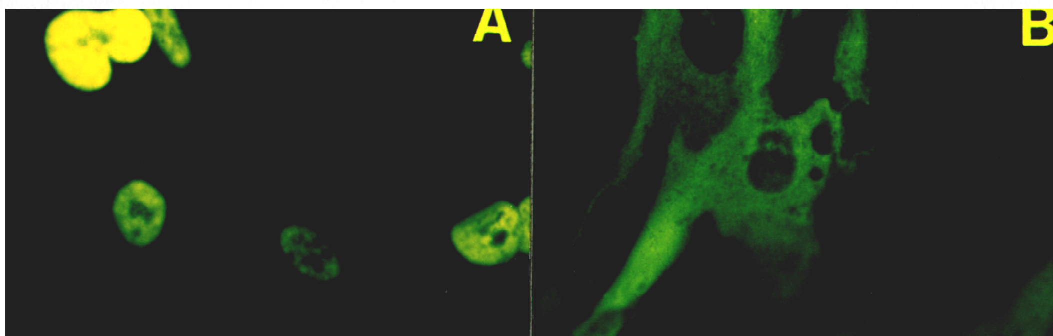

Fig. 4 A) GFP tagged nuclear T antigen protein. **B)** GFP tagged cytoplasmic T antigen protein (Photos courtesy of Dr. F.A. Ray)

In 1984, Kalderon et. al. found that changing the lysine at amino acid position 128 to a threonine (K128T) residue also creates a cytoplasmic protein. In 1994, Woods et. al. tested the ability of this mutant protein to cause chromosomal damage in human diploid fibroblasts. In contrast, they found that D10, the K128T plasmid, causes cytogenetic aberrations at the same magnitude as nuclear T antigen.

Taken together, the research of Ray et. al. and Woods et. al, suggest that the amino acid at position 128 plays a key role in the ability of the T antigen protein to cause chromosome damage in human fibroblast cells. However, a conflict arises because the plasmid constructs used in these two studies were not identical. The aim of the current research is to put the K128T and K128I as well as the wild type T antigen gene into identical plasmid constructs so that the true effect of the single amino acid change may be elucidated. The plasmid construct we have chosen is the pIND expression vector purchased from Invitrogen. pIND allows inducible expression of recombinant proteins in mammalian host cells which have been transfected with the pVgRXR vector. This is a "Ecdysone-Inducible Mammalian Expression System" based on the molting induction system of *Drosophila* which has been altered to induce expression in mammalian cells. "The system uses the steroid hormone ecdysone analog, ponasterone A, to activate expression of the gene of interest via a heterodimeric nuclear receptor" (Ecdysone, Invitrogen, 2002). The research presented in this paper shows the creation of two variations of the pIND plasmid expressing the T antigen protein. One contains the wild type lysine at position 128, while the second contains the K128I mutation. Research will continue in order to obtain the third desired plasmid, which expresses the K128T mutation. Once all three plasmids have been obtained they will be used in future research to determine if, in fact, a particular amino acid at position 128 is necessary for T antigen to cause chromosome damage in human diploid fibroblasts.

Materials and Methods:

<u>Construction of the wild type inducible plasmid:</u>

The inducible plasmid pIND was obtained from Invitrogen (Fig 5). It contains an ampicillian resistance gene, neomycin resistance gene, SV40 origin of replication, and a multiple cloning site in which we have inserted the T antigen gene.

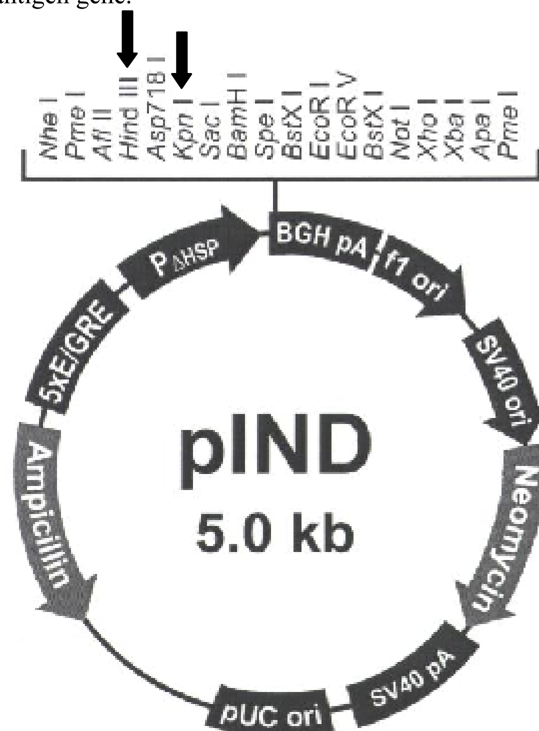

Fig. 5 Map of pIND inducible plasmid obtained from Invitrogen showing key genes as well as the multiple cloning sites. The HindIII and KpnI restriction sites used for the insertion of the T antigen gene are accented with arrows.

The wild type T antigen was taken from the plasmid pRSVT302ΔH2, supplied by Dr. F.A. Ray (Fig.6). This plasmid contains a copy of the T antigen gene from cDNA, so there is no small t antigen gene in the plasmid. Normal T antigen contains two internal HindIII sites, but in this plasmid they have been removed via silent mutation (F.A. Ray, personal correspondence).

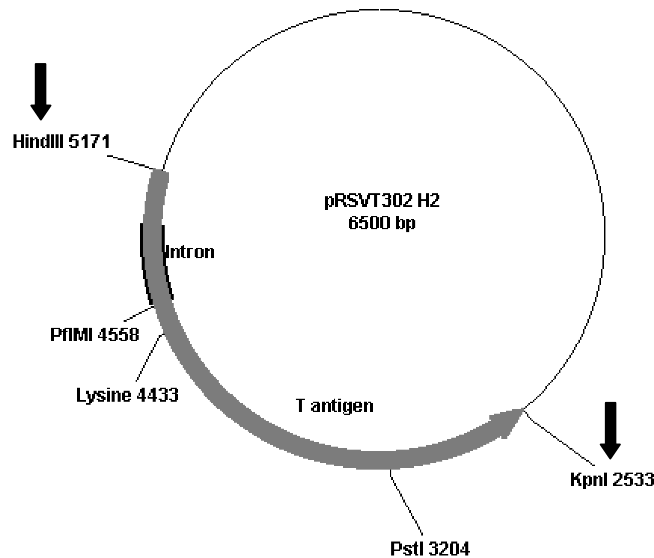

Fig. 6 Map of pRSVT302ΔH2; source of the wild type T antigen gene for this research. The HindIII and KpnI restriction sites used to remove the T antigen gene from the plasmid are accented with arrows.

A HindIII site on the 3' end and a KpnI site on the 5' end flank the T antigen gene. These restriction enzymes were used to cut T antigen from the pRSVT302ΔH2 plasmid, and the DNA band was purified from an 0.8% agarose gel. KpnI and HindIII were also used to open the pIND plasmid. T antigen was inserted into pIND via ligation with T4 DNA ligase. 'OneShot INVαF' chemically competent' *E. coli* purchased from Invitrogen were transformed with the ligation product and plated on LB agar plates treated with ampicillian [100μg/ml].

Construction of the NLS mutant inducible plasmids:

The plasmid pRSVEdl884-14B contains the lysine to isoleucine mutation, which was created by Dr. F.A. Ray (Ray et. al., 1998). This plasmid has the T antigen gene with a deletion of most of the small t antigen region. The plasmid D10 contains the lysine to threonine mutation and was obtained from Dr. S. Bacchetti, though it was created by the Kalderon lab (Kalderon et. al. 1984). The mutation from D10 was inserted into the pRSVEdl884 plasmid construct, creating pRSVEdl884-D10, in January of 2000. The resulting pRSVEdl884-D10 plasmid was used for this study. In these pRSVEdl884 plasmids the mutation occurs at base pair position 2402, and can be easily extracted after a double digest using restriction enzymes PstI and PflMI (Fig. 7). The wild type sequence containing the mutation site was removed from pIND-T, using a complete PflMI digest followed by an incomplete PstI digestion, and the rest of the plasmid was purified from an 0.8% agarose gel (Fig. 8). The K128I mutation was extracted from the pRSVEdl884-14B plasmid constructs using the PstI and PflMI restriction enzymes. The resulting 1354bp band was then purified (Fig. 9) and ligated into the pIND-T plasmid construct using T4 DNA ligase. The product of this ligation was used to transform 'OneShot INVαF' chemically competent' *E. coli.*

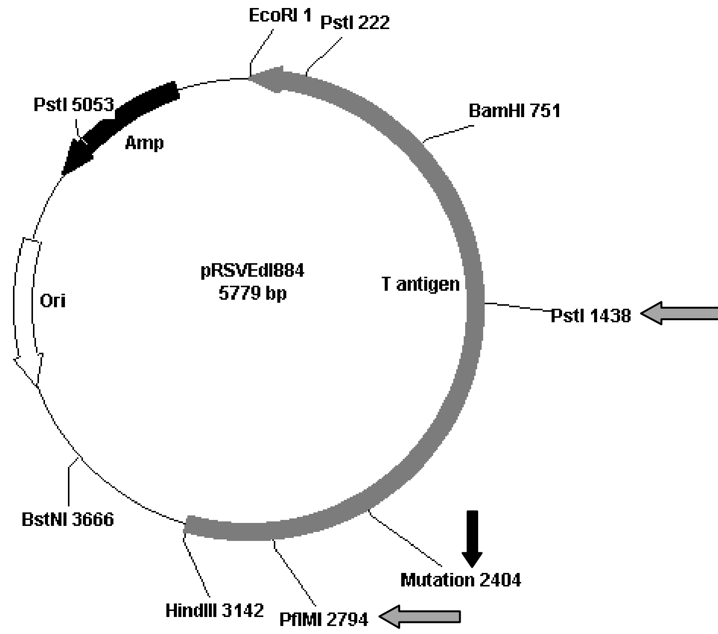

Fig. 7 Map of the pRSVEdl884 mutant plasmids. Note the mutation site (black arrow), and the PstI and PflMI restriction sites (gray arrows).

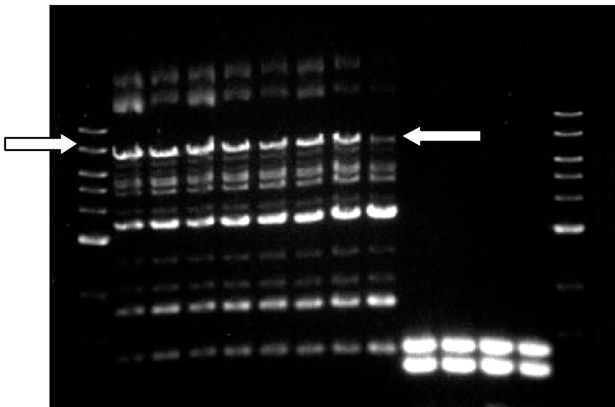

Fig. 8 Analytical agarose gel of the PflMI complete digestion of the pIND-T plasmid followed by incomplete digestion with PstI. The desired band at 6Kb is barely visible in this photo, but is marked with arrows.

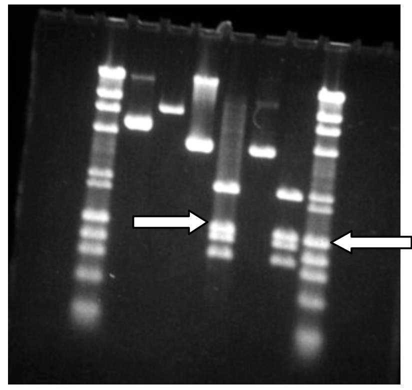

Fig. 9 Analytical gel of the double digestion of pRSVEdl884-14B with PflMI and PstI. The desired band is 1354bp and is marked with arrows.

Results:

Construction of the wild type inducible plasmid:

The colonies, resulting from the ligation of pIND with the T antigen gene from pRSVT302ΔH2, were expanded into 5mL LB media and plasmids were purified using the boiling plasmid quick preparation procedure from Dr. Nickoloff of the Laboratory of Radiobiology at Harvard School of Public Health. This DNA was then digested with multiple restriction enzymes to ensure the desired plasmid had been obtained. After analysis of the resulting agarose gel, the colonies that appeared to have the correct gene insert (Fig. 10a + b), were again expanded, this time to 150mL and the plasmid DNA was extracted using the QIAGEN plasmid purification high-speed midi kit. The plasmid was named pIND-T (Fig. 11).

Construction of the NLS mutant inducible plasmids:

Once the pIND-T plasmid was obtained the 1354bp band between the PflMI and PstI sites was removed from within the T antigen gene, and replaced with the analogous band from pRSVEdl884-14B,

which contains the K128I mutation. The colonies resulting from the transformation of 'OneShot INVαF' chemically competent' *E. coli* were expanded into 5mL LB media, and purified using the QIAGEN mini-prep plasmid purification kit, and the plasmids were digested with multiple restriction enzymes to ensure the desired plasmid had been obtained. After analysis (Fig. 12a+b), the appropriate colonies were again expanded and the plasmids purified.

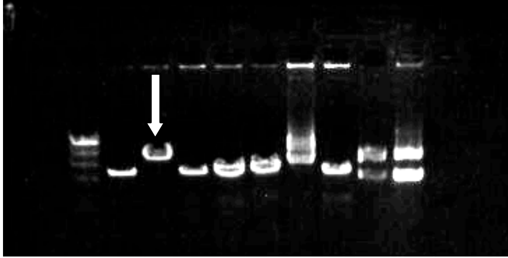

Fig. 10a Analytical gel showing linearized DNA obtained from the plasmid preps of the pIND and wild type T ligation, transformation and resulting colonies. Desired band is 7.3Kb and shown with an arrow.

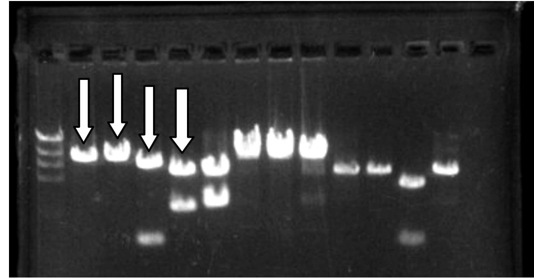

Fig.10b Analytical agarose gel showing diagnostic restriction endonuclease digestions of the plasmid DNA identified in Fig. 11a.
Desired and obtained bands are as follows: lane 2: (BamHI) 7.3Kb, lane 3: (BglI) 7.3Kb, lane 4: (HindIII and BglI) 6 and 1.3Kb, lane 5: (KpnI and HindIII) 5 and 2.3Kb.

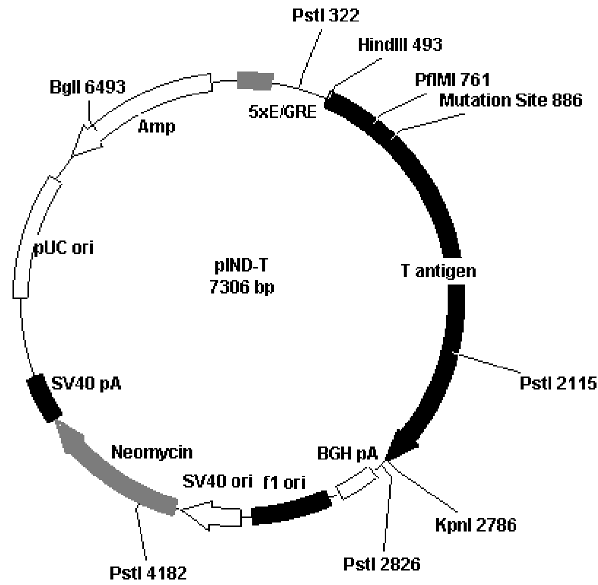

Fig. 11 Map of the pIND-T plasmid

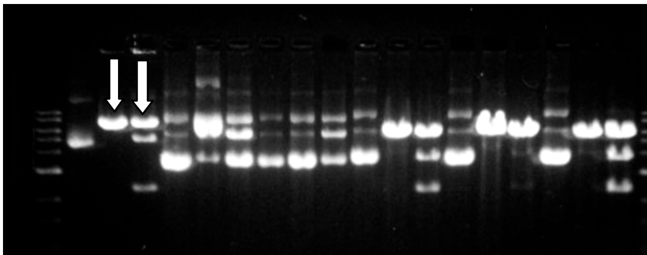

Fig. 12a Analytical agarose gel, showing the electrophoretic bands obtained when pIND-T was digested as follows: lane 3: (BglI) 7.3Kb linear, lane 4: (HindIII and KpnI) 5 and 2.3Kb.

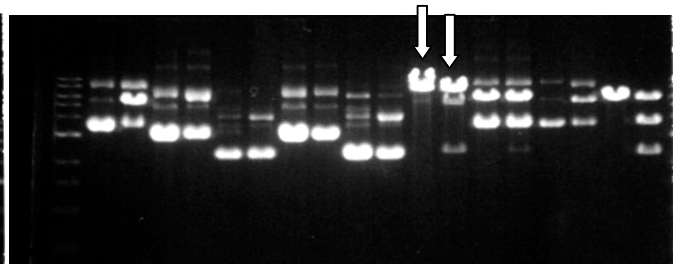

Fig 12b Analytical agarose gel showing the bands obtained from diagnostic digests of DNA purified from *E.coli* colonies resulting from the ligation explained above. Note that the bands match those of the wild-type control from fig. 13a.

DISCUSSION

The SV40 Large T antigen gene alone is necessary and sufficient to cause chromosomal aberrations in human fibroblast cells (Ray et. al., 1990). Since Tag is a nuclear protein with the ability to bind DNA, it was assumed that Tag must bind the DNA in order to cause chromosomal damage. When the NLS is interrupted by a mutation that changes lysine at amino acid position 128 to isoleucine or threonine, the protein becomes cytoplasmic. Ray et. al. found that the K128I mutation causes little chromosome damage, as would be expected of a protein which was unable to access the DNA. Woods et.al. showed that the K128T mutation causes damage at the level of the wild type protein, though it is cytoplasmic (1994). Unfortunately, a confounding problem is that the plasmid constructs used for each of these experiments are different. The goal of the current research was to insert the wild-type Tag and the two mutant Tag genes into the pIND inducible plasmid, in order to ascertain the effect of these single amino acid differences in the context of otherwise identical plasmid constructs. Our results show that the pIND-T, or the inducible plasmid containing the wild type Tag was obtained, and was further utilized to create a plasmid identical in all ways except that the lysine at position 128 was mutated to an isoleucine. These results are based on analytical agarose gel electrophoresis, with subsequent DNA staining with Ethidium Bromide. This type of analysis is based on the inference that the DNA of a given predicted size band is actually the piece expected. There is always the possibility that though the DNA bands we are able to visualize are the appropriate size, they may not actually have the sequence we assume them to have. For this reason, we plan to have the three plasmids sequenced to make sure that they are exactly the same except for one codon, before using them in tissue culture assays. At this time, the K128T mutant pIND-T plasmid has not been created, and work will continue in order to obtain said plasmid. Future research will include sequencing each of the three pIND-T plasmids, as well as tissue culture assays to determine the effects of each of these genes in human fibroblast cells. It is very possible that the confounding results obtained by Woods et. al. and Ray et. al. in terms of the effect of different amino acids at position 128 in the Tag protein are actually caused by some other part of the plasmids used in the respective studies. The plasmid containing the K128T mutation also contains the SV40 promoter and the small t antigen gene, and either of these may play a causal role in the production of chromosome aberrations. Until plasmids possessing lysine, isoleucine, and threonine at amino acid position 128 have been expressed in human fibroblast cells and compared, it is impossible to say that an isoleucine at position 128 prevents the Tag protein from causing DNA damage in human fibroblast cells.

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