THE GENERATION AND CHARACTERIZATION OF L4 - DELETED ADENOVIRUS

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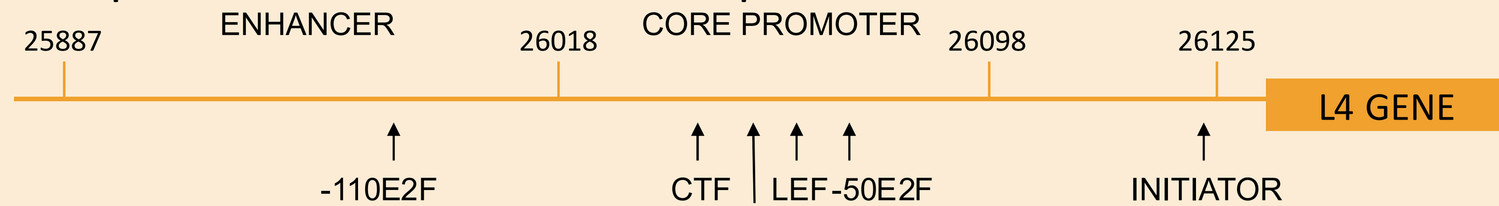
Introduction: Experiment part 1

Adenovirus 5 (Ad5) is widely studied for development as a vector for delivering genes into humans, either for vaccination or gene therapy. Viral genes must be removed to render the vector unable to replicate, and to make space for the desired gene to be added. Genes are controlled by DNA sequences known as promoters. The aim was to create specific mutations in the Ad5 L4 promoter and show their effect on promoter activity. L4 is crucial to successful infection as it produces two proteins, 22K and 33K, that control expression of other viral genes.

The L4 promoter DNA sequence contains several elements that are known to be important for function in other promoters as indicated here:



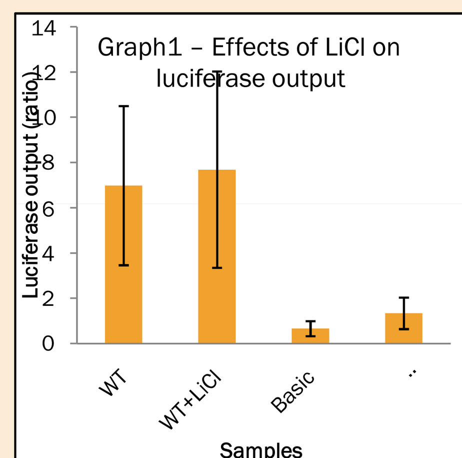
E2F is important for activation of other Ad5 promoters. CTF (CAAT transcription factor) is a general activator of promoters. LEF mediates activation by some cell signalling pathways. The plan was to mutate these sequences to disrupt their function and then test promoter activity using firefly luciferase as a 'reporter gene'.

Methods

1. To achieve the desired mutation, specific primers were used to create DNA fragments by a two-stage polymerase chain reaction (PCR). This fragment was then inserted next to luciferase in a plasmid pBasic 25887-26125 and transformed in *E. coli* cells to produce the mutant plasmids. The successful mutations which were obtained were: Con1, which would serve as a control for the other targeted mutations; -50E2F; CTF.

2. The DNA was transfected into a mammalian cell line (293 cells) and harvested for luciferase and β-galactosidase (control) assays to determine the promoter activity.

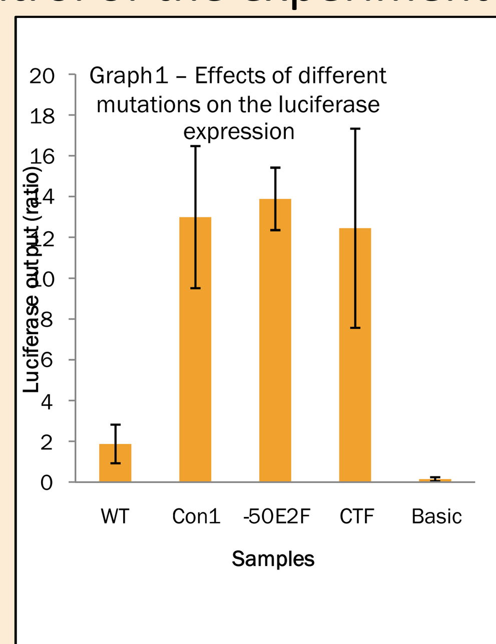
The effects of LiCl on L4 promoter function



Graph 1 compares the unmutated L4 promoter without or with added LiCl. LiCl was hypothesized to activate promoter function via transcription factor binding at the LEF (lymphocyte enhancing factor) site. The theory behind this is that LiCl is an inhibitor to GSK3 protein kinase which prevents the activation of β-catenin, while activation of β-catenin leads to binding of transcription factor at LEF site. This led to the prediction that, if the LEF site was important, LiCl should increase L4 promoter function. Although a small increase is seen in Graph 1, this change was too small to be significant. Due to this result, it was not deemed useful to attempt to make the planned LEF mutant.

The effects of L4 promoter mutations on viral gene expression

The 'WT' (wild type) bar represents the positive control of the experiment as it shows the full activity of the L4 promoter. As for the 'Basic' bar, it is the negative control in the experiment as it contains no promoter at all and should have no luciferase production as shown in Graph 2. However, all the mutant samples gave results completely different from the hypothesis set in the beginning of the experiment. The Con1 mutation was predicted to have no effect on the promoter function but Graph 2 shows that Con1 had a significant increase in luciferase output over WT. Not only this, the -50E2F and CTF mutants, which were predicted to suppress gene expression activity, gave a significant increase in luciferase output indicating substantial elevation in the promoter function.



Conclusion

The generation of mutations in the L4 promoter region was generally successful. Three out of five chosen mutations were successfully created in the experiment. However, the effects of these mutations did not comply with the hypothesis but rather acted in the opposite. This means that identification of the exact location in the L4 promoter which stimulates and enhances gene expression has failed. In order to achieve this aim, new points for mutation need to be determined, new primers designed and the experiment repeated.

Introduction: Experiment part 2

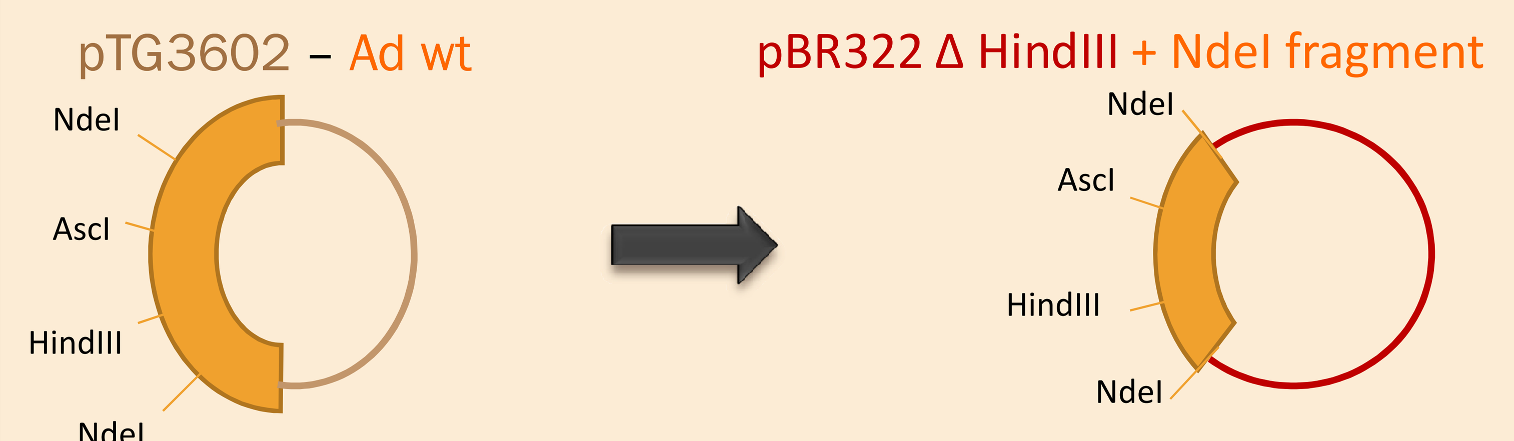
The second part of the experiment aims at the same L4 promoter, however, the mutation which was made was larger – an 80 base pair deletion of the core of the promoter. The objective was to recreate a viral genome carrying this deletion.

In order to achieve this PCR mutagenesis and two different plasmids were used – pBR322 and pTG3602, which had the whole Ad5 genome inserted. The use of the two plasmids was necessary because of the various restriction sites that are on the pTG3602 plasmid. Because there is more than one HindIII restriction site, cutting out the desired fragment would be impossible. This is why a subclone in pBR322 Δ HindIII was constructed.

The expected final product was a virus genome with a non- functioning L4 promoter, which results in the two L4 proteins 22K and 33K not being produced. This would prevent expression of the major late viral proteins.

Methods

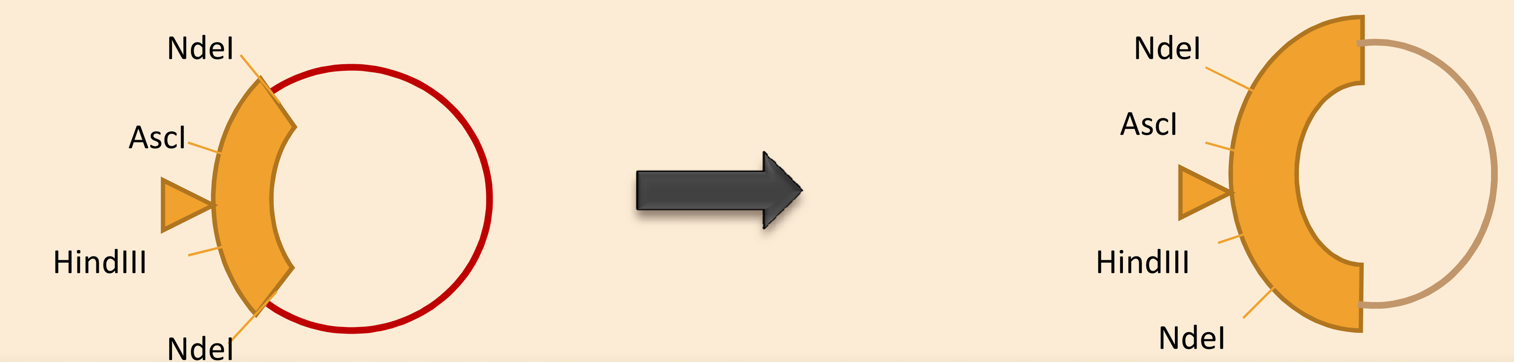
1. Cut out NdeI – NdeI Ad wt fragment from pTG3602 – Ad wt
2. Introduce into pBR322 Δ HindIII



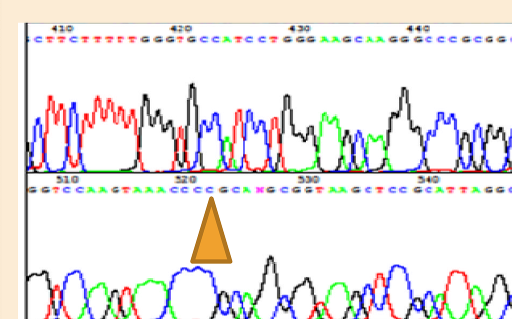
3. Use PCR mutagenesis to delete an 80bp long fragment between AscI and HindIII and so disrupt the L4 promoter

4. Introduce the mutated AscI-HindIII fragment into pBR322 Δ HindIII + NdeI

5. Introduce mutated NdeI fragment back into pTG3602

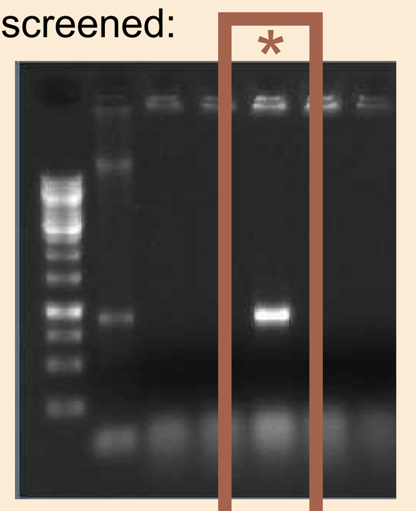


Results



Steps one to four were successful. The deletion was shown to be present in the sequence of one of the clones. Part of the sequence is presented here with the deletion being indicated by the triangle.

Some of the 45 colonies screened:



The first set of results for step 5. is from a colony screen. 45 colonies were screened to find out if the backbone plasmid was pTG3602. The protein V gene from pTG3602 was screened for here. One colony shows to have the pTG3602 backbone (*) by the amplification of the correct DNA fragment by PCR.

CONTROLS
WT MUT

This one positive colony was retested to see whether it contained the fragment with the L4 promoter deletion. However, as we can see the fragment obtained by PCR (*) is the same size as that from a wild type control and larger than the fragment with the deletion. This shows that the insertion into pTG3602 was not successful.

Conclusion

The last step of this experiment was unsuccessful. Therefore no further testing was possible in order to determine whether 22K and 33K would be expressed or not and what effect this has on the expression of the major late proteins. The reason why the backbone would not accept the insert will have to be investigated in order to finish this project.