BIOL5202M Assignment 1: Flow Chart of Figure:

Functional Identification of the Plasmodium Centromere and Generation of a Plasmodium Artificial Chromosome, Iwanaga et al 2010 Figure 2

Hypothesis

Adding PCEN to Plasmids will improve their segregation efficiency during *Plasmodium berghei* Blood-Stage Multiplication

Null Hypothesis – Addition of PCEN to plasmids will have no effect on *P. berghei* Blood Stage Multiplication

Flow Chart

Genes of interest identified **(2a)**

Primers used to select the putative chromosome centromere (PCEN) DNA sequences

DNA Amplified via PCR

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DNA fragments containing the PCENs isolated using gel electrophoresis

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Plasmodium artificial chromosomes constructed. For each PCEN sequence. Schematically shown in **Figure 2a.**

Notes on techniques

- DNA Sequences obtained for pfCEN3, pyCEN5, pbCEN5, pbCEN5A/T, pbCEN5A/T – core, pbCEN5A/T-rep.
- *P.berghei, P.yoelii* and *P.falciparum* strains used for the putative centromere regions. These strains used as active in mice and no risk of infecting human hosts.
- Primers were calculated using DNA as a template from information derived from previous experiments and Figure 1 .
- PCR cycle run to amplify DNA fragments. Exact temperatures for annealing, denaturisation are determined by primer A/T content and thermostable enzyme used. Buffer solution used to provide appropriate enzyme environment.

Gel electrophoresis:

- Mix agarose gel, pour into electrophoresis chamber, add comb and allow gel to set. Ethidium bromide added to DNA. This is a fluorescent dye allows detection of DNA fragments. Remove comb from agarose gel. Load DNA into each well on the agarose gel. Run electric current through for one hour at 90V. Transilluminator used to visualise gel.
- Identify PCEN sequence DNA according to size based on distance travelled through gel.
- DNA extracted from gel using QIA quick gel extraction kit from Qiagen. Followed procedures from kit.

- Each PCEN sequence cloned into digested plasmid to create circular C-PAC.
- NEGATIVE CONTROL - PbGFPcon – plasmid which does not contain pCEN
- cDNA fragments added were digested by Hind III.
- pbGFPcon and PCEN DNA fragments digested with EcoRI
- C-PAC digested with PmeI to remove spacer region between telomeres

Includes: Additional marker genes : GFP (expression) and dhfr-ts (Antibiotic selective marker)

- GFP tag cassette added as expression marker pbGFP additionally digested with EcoRI to remove d-ssu-rna
- dhfr-ts gene for pyrimethamine resistance added from Toxoplsdms
- DNA ligase seals the nick between gene of interest and plasmid vector which creates recombinant vector+plasmid.
- All amplified PCR fragments cloned into PCR2.1 – TOPO vector (Invitrogen)

PCEN Plasmids introduced into P. Berghei schizons.

- To obtain P. Berghei schizons for transfection: Collected 4–16 µl of tail blood from P. berghei–infected mouse.
- PCEN plasmids purified using Qiagen Plasmid Midi Kit (Qiagen).
- Transformation achieved via electroporation to add 5 micrograms of each DNA construct. By applying short electric pulse to the P. Berghei blood stage schizon cells, small pores were created in the cell membrane. Through which the recombinant vector C-Pac containing the PCEN DNA can be easily transferred.
- Amaxa transfection technology used .

Transfected P. Berghei parasites maintained in Swiss Mice at blood stage

Mechanical passage of infected erythoryctes and blood collection for analysis

- The transfection solution (150 µl) is injected using an insulin syringe into a tail vein of a mouse under anaesthesia.
- Two separate transfections were done for each construct to create independent parasite lines.
- P Berghei maintained in Swiss Mice for 1-2 weeks under pyrimethamine drug pressure. Mice provided with drinking water containing pyrimethamine. This kills mosquitoes which do not contain the dhfr-ts gene for resistance via the transfected plasmid.
- Transferred into naive mice for 18-21 days without drug treatment. Mechanical passage done via intraperioneal injection of 2-4 x 10^4 infected erythrocytes 3 to 5 times once parasitemia reached 5-10%.
- 10ul blood collected with each mechanical passage.
- Blood cultured in 1 ml of RPMI1640 medium (pH 7.3) containing 10% fetal calf serum.

Percentage of parasites retaining PCEN constructs calculated using fluorescence microscopy Displayed as **figure 2 B.**

- Samples of infected blood incubated at 37’C for 5 mins in presence of Hoechst 33258 at 10uM final concentration. This stains the parasites nuclei.
- Fluorescence microscopy used to show total number of parasites (Hoeshst)
- Percentage of infected erythrocytes that were GFP positive was assessed using a fluorescence microscope . This identified parasites retaining PCEN and plasmid inserts (GFP).

% GFP calculated and displayed as **Figure 2 C** this shows % of parasites retaining each PCEN sequence

- Calculating the GFP shows what percentage of parasites retained each PCEN sequence.
- This detirmines the minimum region of PCEN needed for sucessful transfection and parasites to survive.
- Calculations compared to what predicted GFP parasites.
- Sequence analysis of the PCENs was carried out using Artemis 10 software

Southern Blot to determine if PCEN plasmids present after multiplication **Figure 2 d.**

- Parasite DNA digested with HINDIII.
- DNA used for each PCEN a) from 7 days multiplication in presence of pyrimethamine and b) DNA from 18 days in absence of pyrimethamine.
- Used SDS page to separate the DNA samples.
- Blots hybridised to P. Berghei 5'UTR dhfr-ts probe.
- Blots were run for pbCEN5, byCEN5, pfCEN3, pbCEN5A/T and bpfGFP con (negative control).
- Probe visualised and hybridization signal compared and quantified using Quality One software (Bio-Rad) to determine the copy number of each plasmid.

Results and Conclusions

Florescence Microscopy demonstrates that parasites infected with pbCEN5 expressed GFP over 18 days after drug pressure removed showing plasmid was retained. **(2b and c)**

Southern Blots of DNA at 7 days and at 18 days showed a single fragment. These matched plasmid sizes demonstrating that the plasmids had not been integrated into the Plasmodium genome. **(2d)**

Control - in parasites transfected with pbGFPcon lacking PCEN the plasmid was lost as shown by no DNA on Southern Blot and no immunofloresence**. (2 b, c and d)**

Additionally PCEN5A/T-rep and PCEN5A/T-core reduction in fluorescence and southern blotting shows that the entire A/T rich centromere region is needed for effective segregation and maintenance of PCEN5 plasmids. **(2, b , c and d)**

References

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JANSE C., RAMESAR J., WATERS A..P. 2006 High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite Plasmodium berghei , *Nature Protocols,* **1**, 346 - 356

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