MOLECULAR ANALYSIS OF ACUTE AND CHRONIC DUCK HEPATITIS B VIRUS (DHBV) INFECTIONS IN DUCKS

Huey Chi LOW

B.Sc. (Biomedical Science), B.Sc. (Hons)

A thesis submitted to the University of Adelaide
in fulfilment of the requirements for the degree of Doctor of Philosophy

Disciple of Microbiology and Immunology
School of Molecular and Biomedical Science
The University of Adelaide
South Australia

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Dedication

To my loving family

who never gave up on me over all these years

Dad

For your silent encouragement and support (financially and emotionally)

Words just simply could not describe how much you mean to me

Mom

For always sacrificing your all to give me the best in everything

I could never ever repay you for all you have done for me

Elder sis

For always being there for me, cheering me up, and trying to help as best as you can

I could not hope for a better, crazier sister than you

This belongs to you all as much as it belongs to me

To God my Heavenly Father be all the glory in the name of His Son

Even youths grow tired and weary, and young men stumble and fall;
But those who hope in the Lord will renew their strength.
They will soar on wings like eagles;
They will run and not grow weary, they will walk and not be faint.
Isaiah 40:31

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Ph.D. Candidate:	Huey Chi LOW
Student ID:	1102743
Ph.D. thesis title:	Molecular analysis of acute and chronic duck hepatitis B virus (DHBV) infections in ducks
Date:	September 2012

Response to Ph.D. Thesis Examination

Brief statement from the candidate

Based on the Thesis Examination Reports, Examiner 1did not specify any amendments to be made to the thesis, while Examiner 2 made extensive comments that needed to be addressed. This document thus details all the amendments made to the Ph.D. thesis in response to Examiner 2's comments.

Examiner 2's comments have been paraphrased from the Report and are shown below underlined. Each comment has been addressed individually. The page numbers referenced in this document are based on the page numbers of the amended thesis, which may vary from the page numbers mentioned by Examiner 2 in the Report (unless stated as "originally pages XX-XX", which refers to the page numbers in the original submitted thesis).

Detailed list of amendments made to the Ph.D. thesis in response to Examiner 2's comments

Layout of thesis

1.1. Include page numbers for Tables and Figures. Page numbers have been added for all Tables and Figures. The respective page numbers have been listed in a separate Table and Contents for Tables and Figures (refer point 2.1).

1.2. Integrate Tables and Figures into the text. Tables and Figures for each Chapter has not been integrated into the text and are placed at the end of each Chapter. This was to avoid disruption of the text, and for easy referencing as all the results were compiled together. To overcome the issue of difficulties in locating the Tables and Figures, page numbers for every Table and Figure have been included (as mentioned in point 1.1).

1.3. Reduce Figure sizes to include legend on the same page. Whenever possible, corresponding legends for Tables and Figures and were put on the same page.

1.4. Points were being repeated in different Chapters. Repeated information in later Chapters were removed and referred to previous Chapters. Some of the changes made have been listed below for each corresponding Chapter.

1.5. Results that were discussed in the Results section would not require a separate Discussion section, but can be finalised with a short conclusion. In all instances, discussion of results was moved from Results and put in the Discussion of each Chapter.

1.6. Layout of the thesis can be improved, e.g. integrate Chapter 5.2 with Chapter 6, and move Chapter 5.3 near to Chapter 6 to solve duplicated information in 6.3.1 and 5.3.4.1. After many considerations and testing of different Chapter/Section rearrangements, the original order of the thesis was still deemed the best and thus was maintained. The duplicated information issue was addressed in point 1.4.

1.7. Spelling out numbers in full when at the start of a sentence, or change the sentence structure so that it doesn't start with a number. All sentences starting with numbers were modified so that the numbers would not be at the beginning of a sentence. For instance, the sentence in Section 2.8.7 (page 55) "1% agarose gel was prepared" was changed to "To prepare 1% agarose gel". Other sentences that were modified include:

a) Abstract (page iv) 75.5%
b) Section 2.8.9 (page 56) 150 ng
c) Section 2.8.9.1 (page 56) 5 µL
d) Section 2.8.9.2 (page 56) 150 ng
e) Section 2.10.2 (page 62) 600 µL
f) Section 2.10.3 (page 63) 10 µL
g) Section 2.10.5 (page 64) 100 µL

h) Section 2.10.6 (page 64) 4 µL
i) Section 2.10.7 (page 64) 40 µL
j) Section 2.11.1 (page 65) 2 sheets
k) Section 2.11.2 (page 66) 50 µCi
l) Section 2.11.2 (page 66) 150 ng
m) Section 2.11.2 (page 66) 4 µL
n) Section 2.11.3 (page 67) 12 mL
o) Section 2.12 (page 67) 1 unit
p) Section 2.12 (page 68) 10 µL
q) Section 2.14.3 (page 72) 20 µL
r) Figure 3.2.7 (page 133) 2
s) Section 4.3.1 (page 137) 1023 bp
t) Section 4.4.4.1 (page 150) 10-fold
u) Figure 4.6 (page 170) 1122 bp
v) Figure 4.9 (page 176) 1052 bp
w) Figure 5.1.4 (page 207) 200 ng
x) Figure 5.1.4 (page 207) 100 ng
y) Figure 5.1.4 (page 207) 300 ng
z) Figure 5.1.4 (page 207) 100 ng
aa) Section 5.2.4 (page 213) 5 ng
bb) Section 5.3.3.2 (page 221) 5 DNA sequencing reactions
cc) Section 5.3.4.2 (page 224) 32 DNA sequencing reactions
dd) Section 5.4.6 (page 247) 1 µg
ee) Section 5.4.6.3 (page 250) 0.5 µg
ff) Section 5.4.6.4 (page 252) 1 µg
gg) Section 5.4.6.4 (page 252) 1 µg
hh) Table 5.4.1 (page 257) [a]5 µg
ii) Figure 5.4.3 (page 261) 5 µg
jj) Figure 5.4.4 (page 263) 5 µg
kk) Section 5.5.3.3 (page 280) 96-well
ll) Section 5.5.3.3 (page 281) 7 DNA sequencing reactions
mm) Section 5.5.3.3. (page 281) 5 DNA sequencing reactions
nn) Section 5.5.4.3 (page 284) 96-well
oo) Section 6.3.4 (page 312) 1.75 µg
pp) Section 6.3.6 (page 313) 37 DNA sequencing reactions
qq) Section 6.3.6 (page 314) 11 DNA sequencing reactions
rr) Section 6.3.6 (page 315) 6 DNA sequencing reactions
ss) Section 6.3.6 (page 316) 18 DNA sequencing reactions
tt) Section 6.3.6 (page 317) 72 DNA sequencing reactions
uu) Section 6.3.6 (page 317) 3 of the 4
vv) Section 6.4.1 (page 318) 12 liver tissues
ww) Section 6.6 (page 326) 5 DNA sequencing reactions
xx) Section 6.6 (page 326) 6 DNA sequencing reactions
yy) Section 6.6 (page 328) 10 DNA sequencing reactions
zz) Section 6.6 (page 328) 15 DNA sequencing reactions
aaa) Section 7.4.3 (page 367) 62 DNA sequencing reactions
bbb) Section 8.5.2 (page 416) 2 of the integrated virus-cell junctions
ccc) Section 8.6 (page 419) 3 integrated virus-cell junctions

1.8. Spell out the symbol "~" as "approximately" when at the start of a sentence. All sentences starting with symbols "~" and "+" were modified. For instance, the symbol in Section 2.6.4 (page 50) "~2 µg" was changed to "Up to 2 µg". Other sentences that were modified include:
a) Section 2.6.6.1 (page 51) ~25mg
b) Section 2.11.1 (page 65) ~500 ng
c) Section 2.12 (page 67) ~2 µg
d) Figure 2.4 (page 83) "+"

e) Figure 5.4.3 (page 261) ~1 µg
f) Figure 5.4.4 (page 263) ~1 µg
g) Figure 5.4.7 (page 269) ~1 µg
h) Figure 5.4.8 (page 270) ~1 µg
i) Section 6.3.2 (page 310) ~150 ng

2. **Table of Contents**

2.1. Add list of Tables and Figures. A secondary Table of Contents for Tables and Figures has been added after the primary Table of Contents and before the Abstract (page i).

3. **Abstract**

3.1. Incorrect formatting for Yang and Summers, 1999. The citation formatting errors for Yang and Summers on pages i, iv, v have been corrected. Other citation formatting errors that were not pointed out, for example Mason *et al.*, 2005 in page vi, have also been corrected.

3.2. Unexplained abbreviation "dpi" (page iii). The abbreviation "dpi" in page iii had already been previously explained in page i.

4. **Chapter 1**

4.1. Add page numbers for Tables and Figures, integrate Tables and Figures into text, reduce Figure sizes to include legend on the same page. These comments are respectively addressed in points 1.1, 1.2 and 1.3 above.

4.2. Quote original authors where possible, and insert "reviewed by..." when quoting review articles. Several citations were changed to the original authors. These include:
a) Section 1.1 (page 1) Lok, 2000 → Blumberg *et al.*, 1967
b) Section 1.4 (page 8) Seeger and Mason, 2000; Jilbert *et al.*, 2008b → Stevens *et al.*, 1975
c) Section 1.4 (page 8) Andersson and Dienstag, 2008 → Beasley *et al.*, 1982
Whenever original articles were not available, "reviewed by..." were added to the citations. These include:
a) Section 1.2.2 (page 2) Kann, 2008
b) Section 1.2.4.1 (page 5) Jilbert *et al.*, 2008a; Yang *et al.*, 2010
c) Section 1.2.4.1 (page 5) Jilbert *et al.*, 2008a; Kann, 2008; Rabe *et al.*, 2009
d) Section 1.2.4.2 (page 5) Kann, 2008
e) Section 1.2.4.3 (page 6) Jilbert *et al.*, 2008a
f) Section 1.4 (page 8) Andersson and Dienstag, 2008
g) Section 1.4.1.2 (page 9) Lok, 2000; Visvanathan, 2008
h) Section 1.4.1.2 (page 10) Bowden, 2008
i) Section 1.4.2.1 (page 12) Funk *et al.*, 2002; Yim and Lok, 2006; Yuen and Lai, 2008
j) Section 1.4.2.2 (page 13) Bowden, 2008
k) Section 1.9 (page 23) Zoulim *et al.*, 2008

4.3. Citation "Jilbert *et al.*, 2008" should be labelled as either "2008a" or "2008b". This error in citation have been corrected in the whole thesis, to correctly cite either Jilbert *et al.*, 2008a or Jilbert *et al.*, 2008b.

4.4. Length of Introduction was rather short, suggest including DHBV natural life cycle, experimental infection of ducks during acute and chronic DHBV infection, duck immune responses e.g. IFN as cytokine production may affect integration events. The overall length of Chapter 1 "Introduction" was not extended from its current length of 27 pages (1.5 paragraph spacing). An Introduction chapter of 25 – 30 pages (double spacing) has been the standard required length for a Ph.D. thesis in the Hepatitis Research Laboratory. Therefore, descriptions of the DHBV natural life cycle in the duck model were not included in Chapter 1, but are briefly described in Section 1.9 (page 24), and described in Chapters 6, 7 and 8 wherever necessary. The possible effects of cytokine/chemokine production on the rate of hepadnavirus integration have been included as a future study direction in Section 9.6 (page 460).

4.5. The ducks' short lifespan (9-12 years) as a possible reason for ducks not developing HCC do not correlate with findings that woodchucks develop HCC within 4 years after infection. It was further clarified in Section 1.9 (pages 24-25) that woodchucks tend to develop HCC within 4

years after WHV infection due to the frequent activation of *N-myc* oncogenes so far only observed in the woodchucks. It was also clarified in Section 1.9 (pages 24-25) that compared to humans and chimpanzees, ducks have a short lifespan.

4.6. A better review of the relationship between HCC development and hepadnavirus integration is needed. The possible relationship between HCC development and hepadnavirus integration has already been addressed in Section 1.8 (page 22) and Section 9.5 (pages 456-458).

4.7. A better review of Section 1.8.1 "Detection of integrated virus-cell junctions" is needed. Section 1.8.1 had been expanded from 232 words to 840 words to briefly explain methods that had been used to detect integrated virus-cell junctions (pages 22-24). Description of Southern blot hybridisation and linker ligation assay was moved to Section 1.8.1 from Sections 8.2 and 5.3.2 respectively.

5. **Chapter 2**

5.1. Integrate Tables and Figures into text, reduce Figure size to include legend on same page, correct sentences that start with number or symbol. These comments are respectively addressed in points 1.2, 1.3, 1.9 and 1.10 above.

5.2. Figure 2.7 is unnecessary if space is a problem. Space was not a significant problem, thus Figure 2.7 (Figure 2.8 in the amended thesis) was retained.

6. **Chapter 3**

6.1. Integrate Tables and Figures into text, reduce Figure size to include legend on same page, correct sentences that start with number or symbol. These comments are respectively addressed in points 1.2, 1.3, 1.9 and 1.10 above.

6.2. Detailed descriptions of the optimization of existing assays are not required. The whole chapter needs to be rewritten and summarised, perhaps by tabulating the results. For Chapters 3.1 and 3.2, most of the results were already tabulated, or not included and merely described in-text if there were not a lot of data. The level of detail included in these Chapters was felt to be needed to justify the amount of work and level of detail scrutinized during the optimization of the DNA sequencing and subsequent cleanup of DNA sequencing products (Chapter 3.1), or during the optimization of qPCR assays for DHBV total and cccDNA (Chapter 3.2). Nonetheless, Chapter 3.1 was shortened from 11 pages (originally pages 61-70) to 10 pages (pages 92-101), while Chapter 3.2 was shortened from 18 pages (originally pages 72-89) to 15 pages (pages 106-120).

6.3. Figures 3.1.2 and 3.2.4 can be deleted. Figure 3.1.2 was omitted and simply described in-text in Section 3.1.4.6 (page 100). Figure 3.2.4 was kept as it showed evidence of over-digestion or non-specific digestions occurring when plasmid DNA were *Eco*RI-digested in the qPCR assay SYBR Green mastermix, something not previously performed and shown.

7. **Chapter 4**

7.1. Integrate Tables and Figures into text, reduce Figure size to include legend on same page, correct sentences that start with number or symbol. These comments are respectively addressed in points 1.2, 1.3, 1.9 and 1.10 above.

7.2. The sentence that "Clustal W2 alignment program was used" is repetitive and can be stated once at the beginning of the chapter. The repetitive sentences were removed from Sections 4.3.2.1 (pages 137-138), 4.4.1.2 (page 141), 4.4.2.2 (page 143), and simply referred to Section 2.8.12 (page 59) which had described the alignment program used in detail.

7.3. Chapter is verbose. Chapter 4 was shortened from 24 pages (originally pages 91-114) to 22 pages (pages 135-156).

8. **Chapter 5.1**

8.1. Too much discussion of advantages and disadvantages for each kit, some of which were repeated in the Results section. The discussion of the advantages and disadvantages of each DNA extraction method used in Section 5.1.3 were retained in order to give a more complete picture in determining the most suitable method to be used in the invPCR assay. The repeated discussions of the DNA extraction methods in the Results Section 5.1.4 (pages 190-199) were removed.

8.2. Section 5.1.4 Long discussion on accuracy of spectrophotometers is not needed. Comparison of Nanodrop spectrometer and Spectromax spectrophotometer in Section 5.1.4 (pages 190-191) was not to determine the accuracy of the Nanodrop spectrometer, but rather to show that this new equipment gives the same readings as the Spectromax spectrophotometer, which has been used in the Hepatitis Research Laboratory for many years. Thus, the results generated in this thesis could be confidently compared and even combined with previously generated results, without the potential differences of greater sensitivity or accuracy of the results obtained using the newer Nanodrop Spectrometry, as claimed by the manufacturer (Thermo Fisher Scientific, Canada). There is no doubt that the Nanodrop spectrometer is accurate. This Section was shortened, and some descriptions of the Nanodrop spectrometer was moved to Materials and Methods Section 2.8.1 (page 53).

8.3. Section 5.1.4.4 is mostly repetitive and can be deleted/summarised. Section 5.1.4.4 (pages 196-199) was one of the more important results for Chapter 5.1, as it looks to quantitate the levels of DHBV DNA and duck genomic DNA present in each extracted DNA sample. The ability to extract large amounts of duck genomic DNA with the least amounts of DHBV DNA is one of the main criteria for selecting a DNA extraction method to be used in the invPCR assay. Hence this Section was not removed. It was shortened from 1513 words (originally pages 132-135) to 1188 words (pages 196-199).

8.4. Detailed descriptions of the optimization of existing assays are not required and can be summarized in a table of results. Entire Chapter 5.1 was revised to be more succinct, with repeated information removed in subsequent paragraphs. Chapter 5.1 was shortened from 22 pages (originally pages 115-136) to 19 pages (pages 181-199).

9. **Chapter 5.2**

9.1. Some information in introduction has been repeated in discussions and thus can be removed. Entire chapter was revised to be more succinct, with repeated information removed in subsequent paragraphs. Chapter 5.2 was shortened from 8 pages (originally pages 137-144) to 7 pages (pages 210-216).

10. **Chapter 5.3**

10.1. Section 5.3.3.3 direct comparison of the linker ligation assay and the invPCR assay cannot be made as liver used was from different woodchucks. Direct comparison of the methods on the same liver samples was unfortunately not able to be performed due to the limited availability of in liver samples from the woodchucks. Instead, two woodchucks were chosen based on their similar WHV infection outcome and viral load, for as close a comparison as allowed. The discussion in Section 5.3.3.3 (page 222) was modified from a direct comparison of number of actual integrated virus-cell junctions detected, to the trend of the junctions detected. Statements were also included to note that both assays were compared using different liver samples.

10.2. Section 5.3.4.3 Provide details and reference to results of duck tissues mentioned here. Each duck invPCR assay results from Chapter 6 that was discussed in Section 5.3.4.3 was specifically referenced (pages 225-226).

10.3. Comment on the woodchuck and duck BLAST homology of integrated sites that included non target species homology. Statements were included in Section 5.3.3.2 (page 221) and 5.3.4.2 (pages 224) in regards to the BLAST homology to the insert DNA were not from the target species. This was due to the woodchuck and duck genome being not fully sequenced, and thus an insert DNA BLAST homology to other mammalian or avian species respectively would also be accepted. On a similar note, as the human genome has been fully sequenced and made accessible on GenBank, invPCR assay studies in humans would only accept any insert DNA with BLAST homology to humans as a true integration junction. InvPCR assay studies in chimpanzees similarly would also accept any insert DNA BLAST homology to humans as a true integration junction, based on the close evolutionary relationship between chimpanzees and humans (mentioned in Section 5.5.4.3 page 285).

10.4. Introduction Section 5.3.2 was shortened from 3 pages (originally pages 145-147) to 2 pages (pages 219-220), with discussions regarding the linker ligation assay moved to Introduction

Section 1.8.1 (pages 22-24). The entire Chapter was revised to be more succinct, with repeated information removed in subsequent paragraphs. Chapter 5.3 was shortened from 10 pages (originally pages 145-154) to 8 pages (pages 219-226).

11. **Chapter 5.4**

11.1. The entire Chapter was revised to be more succinct, with repeated phrases/sentences removed in subsequent paragraphs. Chapter 5.4 was shortened from 21 pages (originally pages 155-175) to 19 pages (pages 238-256).

12. **Chapter 5.5**

12.1. Section 5.5.4.1 Error in reporting that chimpanzee was 22 years old with history of chronic HBV for 27 years. Chimpanzee 4x222 was corrected to be "44 years old" at the time of biopsy (page 284).

12.2. Insertion of published papers. Hard copies of the two papers arising from contributions of results obtained in this Chapter were included in a pouch attached to the final bound Ph.D. Thesis. Soft copies (PDF files) of the two papers were also included in the CD containing the softcopy of the final Ph.D. Thesis.

12.3. Statement was included in Section 5.5.3.3 (page 281) that due to the woodchuck genome being not fully sequenced yet, for the woodchuck invPCR assay, an insert DNA BLAST homology to other mammalian species would be accepted. Another similar statement was included in Section 5.5.4.3 (page 285) that due to the close evolutionary relationship of chimpanzees and humans, for the chimpanzee invPCR assay, an insert DNA BLAST homology to either chimpanzee or human would be accepted.

12.4. Entire chapter was revised to be more succinct. Repeated information regarding invPCR assay and integration of viral dslDNA in Section 5.5.3.2 (page 280) were removed and referenced to Section 1.8.1 (pages 23-24). Chapter 5.5 was shortened from 10 pages (originally pages 177-186) to 8 pages (pages 279-286).

13. **Chapter 6**

13.1. Section 6.3.1 is a repeat of Section 5.3.4.1. The repeated information was removed (page 309) and reference back to Section 5.3.4.1 (page 223).

13.2. Section 6.3.2 Elaborate on whether the base pair differences of the DHBV sequences detected were near the 5' and 3' end and thus may affect the ability to detect integrated DHBV DNA. As shown in Figure 6.1 and described in Section 6.3.2, most mutations were at the first 35 nucleotide of the sequenced DHBV, one mutation was at nucleotide 502, and the rest were at the last 20 nucleotide of the sequenced DHBV. A statement was added in Section 6.3.2 that restriction enzymes for the duck invPCR assays should not have digestion sites at nucleotide 502, but the duck invPCR assay PCR primers could be designed to amplify across nucleotide 502 (pages 310-311). Based on Figure 6.4, for the chosen duck D3 invPCR assay design, the restriction enzyme digestion sites are at nucleotides 391, 505 and 526, while primer binding sites range from nucleotides 172 – 231 and 465 – 509. Thus, the mutations found in the DHBV sequences should not affect the restriction enzyme digestions, while PCR amplification should not be affected with a single base pair change at the primer binding site of nucleotide 502, and therefore would not affect the invPCR assay to detect integrated DHBV DNA.

13.3. Include headings for Table 6.3 with more information on the duck samples used. Headings with general information of the groups of ducks tested in this Section were added to Table 6.3 (page 337). Detailed explanations of each type of vaccination, antiviral treatment, time-points of liver biopsy, viral load etc. were not included, as the details of each duck were already mentioned in-text and referred to in the table legend, and would clutter and confuse Table 6.3 when included in the table heading.

13.4. Section 6.4.2.2 Query whether the invPCR assay was repeated for ducks FN76, FN77, FN78. A statement has been added to clarify that the invPCR assays had been repeated multiple times, all achieving similar results, which were no detectable invPCR products with only smears as seen by agarose gel electrophoresis (page 323).

13.5. Section 6.4.2.2 Query whether more work should be done in ducks FN1 and FN3 to determine the effect of antiviral therapy on viral integration. No further analysis of FN1 and FN3 liver samples were performed in this thesis, although future work could continue to analyse these liver samples to observe the possible effects of antiviral therapy on viral integration, clonal proliferation of hepatocytes, and formation of hepatocyte clones. A statement was added in Section 6.4.2.2 (pages 322-323) to note that "no further analysis was performed in this thesis although future work could continue to analyse these liver samples to observe the possible effects of antiviral therapy on viral integration, clonal proliferation of hepatocytes, and formation of hepatocyte clones".

13.6. Section 6.6 Commented that invPCR results seemed variable, especially the DNA sequencing failure rates. Query whether Dr. Mason used exactly the same assay and methods, if so it would be the optimised methods. The methods performed in this thesis were initially slightly different from the methods used by Dr. Mason, due to the different availabilities of equipment in each lab (e.g. plate clamp, plate centrifuge), and potential differences in the quality of reagents used in each lab. Many strategies were suggested in Section 6.6 (pages 325-330) to improve the results obtained from the Hepatitis Research Laboratory. But it is true that sometimes some "batch variations" of invPCR assay results do occur at times, e.g. one batch of sequencing with have significantly more failed results compared to normal. In general however, sequencing reactions performed by Dr. Mason do have less failed results compared to sequencing reactions performed in this thesis.

13.7. Compare results using a table. Presentation of the results obtained from this thesis and from international collaborator Dr. William Mason as a table was not added. Although a table would definitely allow for direct comparison of both studies, there was too much information that would need to be included due to the many different samples and conditions of invPCR assays performed, causing the suggested table to be long and confusing.

13.8. Statement was included in Section 6.3.6 (page 313) that due to the duck genome being not fully sequenced yet, an insert DNA BLAST homology to other avian or mammalian species would also be accepted.

13.9. Entire chapter was revised to be more succinct. Chapter 6 was shortened from 27 pages (originally pages 187-213) to 24 pages (pages 308-331).

14. **Chapter 7**

14.1. The phrase "as there were no control ducks in this study..." was repeated numerous times. The control ducks used in this Chapter was described once in Section 7.3.1 (page 353), and removed from subsequent paragraphs, which only referred back to Section 7.3.1. All the other repeated information throughout the Chapter, like Section 7.3.2.9 (originally pages 231-232) that was repeated in Section 2.8.9.2 (page 57), was removed and referenced back to the corresponding previous Sections.

14.2. Table 7.1 The legend is too long. The histological grading system was moved to Section 2.4.1 (page 44), and simply referred to in the legends of Tables 7.1 (page 372).

14.3. Section 7.3.2.2 Query whether the ducks in this study were kept together as a group. If so, all ducks would be exposed to the same environmental toxins. All ducks in this study were kept together in the same housing pen. Explanations were added that the duck 342 with higher levels of liver injury due to environmental toxin was not due to exposure of only that duck in the whole group to the toxins, but rather the potential higher sensitivity and reactivity of that duck towards the toxins, compared to the other ducks (page 356).

14.4. Figure 7.5A DHBsAg levels were above the limit of detection between 25 and 36 dpi, but was referred to in Section 7.3.2.5 that they were below the limit of detection. The cut-off lines in Figures 7.5A, 7.5B and 7.5C (page 388) were misplaced. The positions of the cut-off lines have been corrected, and now correctly showing that the levels of DHBsAg were always below the level of detection throughout the study, as described in Section 7.3.2.5 (page 361)

14.5. Section 7.3.2.8 Comment that Vickery and Cossart (1996) J. Hepatology 25:504 found that peak viraemia could be delayed in older ducks until 15 dpi. Vickery and Cossart's published study (1996) inoculated 26-day-old ducks with DHBV, which were much younger than the 42-day-old ducks used in this study. As the ducks in Vickery and Cossart (1996) were not age

matched, they were therefore not used for comparison in this Chapter. To emphasise that the comparisons made were with published age matched ducks only, the description "previously published age matched ducks" was included (page 363).

14.6. Move Section 7.6 to Chapter 9. The discussions of future directions from Section 7.6 (originally pages 239-243) were merged with Section 8.6 (originally pages 270-275) and moved to Sections 9.6 and 9.7 (pages 456-466), and replaced with a short "Conclusion" (pages 370-371).

14.7. Figure 7.7 (representation of primer binding sites on AusDHBV rcDNA) was moved to Chapter 2 as Figure 2.4 (page 83), as it was deemed more appropriate for this information to be placed in the Materials and Methods Chapter 2, which describes the qPCR methods and primers used for the amplification of the AusDHBV rcDNA.

14.8. Entire chapter was revised to be more succinct. Chapter 7 was shortened from 29 pages (originally pages 215-243) to 23 pages (pages 349-371).

15. **Chapter 8**

15.1. Section 8.2 Correction that male birds are homozygous for the sex chromosome, and not females as in the case for mammals. A statement clarifying that homozygous sex chromosomes are found in mammalian females but in avian males was added (page 398).

15.2. Chapter has repeated points from Chapter 7, e.g. Section 8.2 detection of integrated virus-cell junctions, Section 8.3.2.4 Kupffer cells and their staining, Section 8.4.1 reason for sequencing the DHBV genome, Section 8.5 reason for low detection rate of integrated virus-cell junctions etc. All repeated explanations, like in Sections 8.3.2.1 to 8.3.2.9, Section 8.4.1, Section 8.5.1, and Section 8.5.2 that had been previously mentioned in Chapter 7 were removed, and replaced with corresponding references to Chapter 7.

15.3. The phrase "as there were no control ducks in this study..." was repeated numerous times. The control ducks used in this Chapter was described once in Section 8.3.1 (pages 399-400), and removed from subsequent paragraphs, which only referred back to Section 8.3.1.

15.4. Section 8.4.3 typo that "4 integrated virus-cell junctions" should be "3". Typo was corrected to "3 integrated virus-cell junctions" (page 412).

15.5. Section 8.5.2 Comment that preliminary work was done on neonatal ducks and thus may behave differently from this study. A note was added that the reported rate of DHBV integration published by Yang and Summers (1999) was obtained from a study of young 6-day-old ducks, and thus may be different to the rate of DHBV integration of 6-week-old ducks in this study (page 414).

15.6. Move Section 8.6 to Chapter 9. The discussions of future directions from Section 8.6 (originally pages 270-275) were merged with Section 7.6 (originally pages 239-243) and moved to Sections 9.6 and 9.7 (pages 456-466), and replaced with a short "Conclusion" (pages 418-419).

15.7. Section 8.5.2 Include more explanations addressing the reasons why Dr. Mason detected more integrated virus-cell junctions compared to this study. The reason that the "invPCR assay is not optimised" is not sufficient. Additional discussions regarding the differences of invPCR assays performed in this thesis compared to invPCR assays performed by collaborator Dr. Mason in the USA, that may have affected the low detection of integrated virus-cell junctions in this thesis, were included (page 417).

15.8. Section 8.5.2 Comment that Australian ducks may have differences in genetic makeup due to breeding in isolation for many generations, thus may affect the rate of viral integration compared to ducks from elsewhere in the world. Additional comment on the possible effects of the genetic makeup of ducks in this Australian study due to isolated breeding on the rates of DHBV integration into the genome have been added (page 416).

15.9. Link natural history of hepatitis in the various species to the quantity of integration sites detected. The ducks' short lifespan as the reason for less integration junctions do not correlate with findings that woodchucks seemed to have the most integration sites in the same infection duration with ducks. Additional comments on the differences of the natural history of DHBV infection in ducks compared to other hepadnavirus infections in their natural hosts, in respect

to the different rates of DHBV integration into the genome and of clonal proliferation of hepatocytes have been added (pages 419-420).

15.10. The cut-off lines in Figure 8.5C (page 436) were misplaced. The positions of the cut-off lines have been corrected.

15.11. Entire chapter was revised to be more succinct. Section 8.2 (pages 397-399) was summarised and shortened, with details of the invPCR assay moved to Introduction Section 1.8.1 (pages 22-24). Chapter 8 was shortened from 31 pages (originally pages 245-275) to 23 pages (pages 397-419).

16. **Chapter 9**

16.1. Sections 7.6 and 8.6 "Future directions" were combined and summarised as new Sections 9.6 (pages 459-462) and 9.7 (pages 463-466).

17. **References**

17.1. PNAS should be shortened to Proc. Nat. Acad. Sci. This journal is commonly shortened as Proc. Nat. Acad. Sci. However, the abbreviation PNAS was still preferred in the context of this thesis, and have been defined in Abbreviations page xviii. Other journals, including the European Molecular Biology Organization (EMBO) and Public Library of Science (PLoS), have also been similarly abbreviated and defined in Abbreviations pages xv and xviii respectively.

Other amendments made to the Ph.D. thesis

1. The entire thesis was reviewed several times thoroughly and in greater depths to correct any typos, incorrect cross references, formatting errors, unexplained abbreviations, grammatical errors, and citation formatting errors that were not pointed out by the Examiners. The list of all changes made are not included in this document, as all the changes were minor and did not alter or affect any information or layout of the thesis.
2. The Declaration of originality and consent (page vii) was updated to the new Thesis Declaration as of July 2012 that was released by the Adelaide Graduate Centre, the University of Adelaide.
3. Section 1.3 "Animal models for the study of HBV" was moved to become Section 1.9 (pages 24-26) for a better flow in the Introduction Chapter 1.

Table of Content for Tables and Figures

Abstract

The hepadnavirus family contains two genera, Orthohepadnaviruses that infect mammals and Avihepadnaviruses that infect birds. The most thoroughly studied members of the hepadnavirus family include hepatitis B virus (HBV), which infects humans and chimpanzees, woodchuck hepatitis virus (WHV), which infects woodchucks, and duck hepatitis B virus (DHBV), which infects ducks. All hepadnaviruses have similar genome structure and organisation of the open reading frames on their genome, and also show extensive nucleotide sequence homology within, but not between, genera. All hepadnaviruses primarily infect hepatocytes and have similar infection outcomes in their natural hosts. The outcome of hepadnavirus infection can be divided into either acute, with successful clearance of infection from the host and establishment of immune control, or chronic, where infection is not cleared, resulting in persistent lifelong infection that is linked to various forms of liver disease.

The mechanisms involved in immune clearance of hepadnavirus infections are poorly understood. It has been hypothesised that virus DNA and proteins are, 1) cleared from the liver cytolytically by killing of the infected hepatocytes, or 2) can be destroyed non-cytolytically by cytokines, leaving the infected hepatocyte intact. In this study, the mechanism of immune clearance was explored using molecular approaches to detect integrated virus-cell junctions, formed by the integration of DHBV DNA into the duck liver genome. The rate of DHBV DNA integration is thought to be low (reported to occur at a rate of ~1 in $10^3 - 10^4$ cells by 6 day post infection (dpi)) (Yang and Summers, 1999), and the site of viral DNA integration into the host cell genome is random. Hence, each integration event will produce a unique integrated virus-cell junction that can be used as a genetic marker for that cell, which allows the fate of the DHBV-infected hepatocytes to be observed in the liver during various stages of both acute and chronic DHBV infection. Integrated virus-cell junctions can be detected using inverse nested PCR (invPCR), as described by Yang and Summers (Yang and Summers, 1999). Thus, this study aimed to determine whether cytolytic or non-cytolytic clearance of infected hepatocytes occurred, with the ultimate aim of contributing knowledge to assist in the development of new therapies for patients with chronic HBV infection.

Initially, several molecular assays required for the characterisation of DHBV infection and the invPCR assay were established and optimised. These included DNA sequencing reactions and subsequent purification of DNA sequencing products (Chapter 3.1), quantitative PCR (qPCR) assays used for the detection of DHBV total and cccDNA (Chapter 3.2), as well as cloning and sequencing of the duck beta actin (β actin) and glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) genes. The latter were both used as internal controls in the qPCR assays to quantify levels of DHBV total and cccDNA per cell (Chapter 4). Several parameters were tested and compared for each molecular assay to generate final optimised protocols, which were then applied both throughout subsequent Chapters and in other work performed in the laboratory. The duck β actin and GAPDH genes were characterised and the sequences were submitted to GenBank (GenBank Accession GU564232 and GU564233 respectively).

The invPCR protocol was optimised by comparing 8 different liver cell DNA extraction methods, to determine the method that most efficiently extracted high molecular weight (MW) DNA from small quantities of frozen liver (Chapter 5.1). One of the major problems with the invPCR assay was the high level of virus-virus (false positive) sequences generated from replicative forms of virus DNA. Therefore, the use of locked nucleic acids (LNA) oligonucleotides that were specific for the DHBV genome were tested as a potential selective tool to block the amplification of DHBV DNA (Chapter 5.2). LNA oligonucleotides can bind with high affinity to their complementary DNA sequence, forming high melting temperature LNA-DNA complexes which then block amplification of that DNA sequence. Although the LNAs successfully blocked amplification of virus DNA, due to the high cost of LNA, their use in invPCR assays could not be pursued in this thesis. As a possible alternative to the invPCR assay, a linker ligation assay was assessed for its ability to detect integrated virus-cell junctions in samples of WHV-infected woodchuck and DHBV-infected duck liver tissue (Chapter 5.3). However, as the efficiency of detection of integrated virus-cell junctions using linker ligation assay was similar to the efficiency of the invPCR assay, it was not further developed in this thesis, as the linker ligation assay is more complicated and time-consuming than invPCR.

After optimising all the required molecular assays required for the invPCR assay in previous Chapters, the invPCR assay was first performed on plasmid DNA constructs as model systems, in order to determine the sensitivity and efficiency of the assay (Chapter 5.4). It was found that the invPCR assay was able to amplify from a single copy of DNA template. The invPCR assay was also performed on samples of WHV-infected woodchuck and HBV-infected chimpanzee liver tissue in collaboration with Dr. William Mason (Chapter 5.5). The results of the invPCR assay results confirmed that the assays established in this study had comparable efficiency with previously published invPCR assays, and could be confidently applied in this thesis. The integrated virus-cell junctions obtained in this study were included as a subset of the data published by Mason *et al.* 2009a and 2009b.

In order to establish an invPCR assay specifically for the AusDHBV strain (GenBank Accession AJ006350), several DHBV invPCR assays were designed, and preliminary studies were performed on liver tissues collected previously in the laboratory from ducks with acute and chronic DHBV infection (Chapter 6). Up to 38 integrated virus-cell junctions were successfully detected, among which 3 junctions were repeated, suggesting that clonal proliferation of hepatocytes had occurred in the duck liver. Characterisation of the integrated DHBV DNA showed that, consistent to previous studies, the most probable left and right hand end of the integrated DHBV dslDNA was at nucleotide position 2537 nt and ~2485 nt respectively, with no significant patterns or apparent preference of DHBV integration sites in the duck genome. The duck invPCR assay was subsequently extensively used in Chapters 7 and 8.

To achieve the main aim of this thesis, and to explore if cytolytic or non-cytolytic clearance occurs during the resolution of acute hepadnavirus infection, the invPCR assay was used to detect integrated virus-cell junctions during and after resolution of acute DHBV infection. This DHBV model provided an advantage over the WHV-infected woodchuck and the HBV-infected chimpanzee models, where hepadnavirus infections spreads to > 95% of the hepatocytes in the liver. Widespread infection requires the division of infected hepatocytes during clearance to replace hepatocytes killed by the host immune response. But in the duck model, DHBV infection can be established in ducks by inoculation with defined doses of DHBV to achieve a maximum DHBV infection of <10% of the hepatocytes prior to immune clearance. Therefore, when the few DHBV-infected hepatocytes were killed, they would be most likely be replaced by division of uninfected hepatocytes. It is thus expected that if cytolytic hepatocyte killing was the predominant response to DHBV infection, the integrated virus-cell junctions would not be detected in the recovered liver, as the infected hepatocytes would be destroyed and replaced by previously uninfected hepatocytes. Conversely, if non-cytolytic hepatocyte curing was predominant, the integrated virus-cell junctions would still be detected after clearance of infection as they would be present in the cellular genome of the "cured" hepatocytes.

Using AusDHBV, acute DHBV infections were established in 3, 6-week-old ducks (Chapter 7). At 4 dpi, active DHBV replication was occurring (159 and 3.6 copies of DHBV total and cccDNA per infected hepatocyte) with ~4.4% of DHBV surface antigen (DHBsAg)-positive hepatocytes in the liver. At 45 dpi, <0.001% of hepatocytes were DHBsAg-positive, with residual DHBV DNA predominantly in the cccDNA form (~0.02 copies and ~0.01 copies of DHBV total and cccDNA per liver cell). Histological characterisation of liver tissues showed

elevated levels of portal and lobular liver inflammation, hepatocyte apoptosis, and Kupffer cell infiltration and activation during clearance of infection (4 and 18 dpi), returning to "basal" levels after clearance (45 dpi). Serological characterisation revealed anti-DHBc antibodies by 4 dpi and anti-DHBs antibodies by 11 dpi. The neutralising anti-DHBs antibodies would have removed DHBV virions from the serum by forming antibody-antigen complexes to reduce the levels of viraemia, as only $2x10^6$ copies of DHBV DNA was detected per mL of serum at 11 and 14 dpi. The anti-DHBs antibodies were also continuously produced at high levels after resolution of infection (45 dpi), providing protection against further DHBV infection.

Using the invPCR assay, up to 24.3 µg of DNA was analysed from liver tissues at 4, 18 and 45 dpi. However, no integrated virus-cell junctions were detected at any stages of acute DHBV infection out of the 286 invPCR products sequenced. Up to 75.5% of the invPCR products sequenced were found to be virus-virus (false positive) sequences. As DHBV DNA integration has been reported to occur at a rate of ~1 in $10^3 - 10^4$ cells by 6 dpi (Yang and Summers, 1999), out of the $9.7x\ 10^6$ cells analysed in this study, ~ 4.4% of which were DHBsAg-positive, it was expected that at least 40 – 400 integrated virus-cell junctions would be detected, assuming that the DHBV-infected hepatocytes were not cytolytically killed during resolution of infection. The failure to detect integrated DHBV-cell junctions in these studies could be due to several factors. Firstly, the high levels of invPCR assay false positives contributed by virus-virus sequences detected in this study (75.5% of total invPCR products) may have greatly reduced the efficiency of detection of the integrated DHBV-cell junctions. This could be improved by further optimisation of the invPCR assay, as well as the development of assays to reduce the detection of virus-virus sequences. Secondly, as the rate of integration was obtained from studies with ongoing high levels of active viral replication at ~6 dpi, the actual rate of integration in this study may be much lower than expected, and as DHBV infection were cleared by 18 dpi, this further shortened the timeframe for integration events to occur.

As an extension to the aims of this thesis, the molecular assays established in Chapters 3 to 6 were applied to study events occurring during chronic DHBV infection in ducks. In particular, the histological and serological changes occurring during different stages of chronic DHBV infection were characterised. In addition, changes in the hepatocyte population were studied by detecting integrated virus-cell junctions using the invPCR assay. As the frequency of the integrated virus-cell junctions would increase upon clonal proliferation of the infected hepatocytes, the size of hepatocytes clones formed and the rate of clonal proliferation of

hepatocytes could be determined by measuring the number of repeats for each integrated virus-cell junction. By establishing chronic DHBV infection in 6-week-old ducks, it was expected that chronic DHBV infection may be associated with increased levels of immune attack resulting in elevated levels of liver inflammation, hepatocyte apoptosis and activated Kupffer cells, as compared to ducks infected, for example, at 1 day of age. With a higher rate of immune attack and liver cell turnover, it was expected that hepatocyte clones would be formed in the liver by clonal proliferation of hepatocytes, as seen previously in studies performed in chronic hepadnavirus infection in humans, chimpanzees, and woodchucks.

Using the same AusDHBV virions, widespread and persistent DHBV infections (termed chronic DHBV infections) were established in 3, 6-week-old ducks (Chapter 8). At 4 dpi, active DHBV replication was occurring (219.5 and 3.7 copies of DHBV total and cccDNA per infected hepatocyte) with ~4.1% of DHBsAg-positive hepatocytes in the liver. At 18 dpi, the DHBV infection spread to >95% of hepatocytes in the liver, and was maintained until 165 dpi (duck 343) or 327 dpi (ducks 338 and 350), with continuous high levels of DHBV replication (181 – 298 copies of total DNA per liver cell) and viraemia (0.6 – 20 μg/mL DHBsAg; $1x10^6$ – $1x10^9$ copies of DHBV DNA per mL of serum), and the establishment of a pool of 5 – 9 copies of DHBV cccDNA per liver cell. Histological characterisation of liver tissues at 4 dpi showed elevated levels of portal and lobular liver inflammation and hepatocyte apoptosis, but with low levels of Kupffer cell infiltration and activation. By 18 dpi, widespread DHBV infection and marked portal and lobular liver inflammation and elevated levels of hepatocyte apoptosis and Kupffer cell infiltration and activation were observed in the liver. From 60 – 327 dpi, the levels of hepatocyte apoptosis and Kupffer cell activation persisted, while levels of liver inflammation and Kupffer cell infiltration decreased. Serological characterisation revealed anti-DHBc antibodies from 4 dpi, and anti-DHBs antibodies from 4 – 11 dpi, at low and varying titres. However, the anti-DHBs antibodies were not sufficient to complex to and remove the DHBV virions from the serum, as "active" viraemia was also detected, indicating that the levels of circulating DHBV virions in the serum were in excess of the anti-DHBs antibodies.

Using the invPCR assay, up to 22.8 μg of DNA were analysed from liver tissues at 4, 18 and 60, 165, 196, 327 dpi. Out of the 685 invPCR products sequenced, 3 integrated virus-cell junctions were detected, but no hepatocyte clones were found. 81% of the invPCR products sequenced were found to be virus-virus (false positive) sequences. As mentioned previously, integration of DHBV DNA has been reported to occur at a rate of ~1 in 10^3 – 10^4 cells by 6 dpi (Yang and Summers, 1999), thus out of the ~ $8x10^6$ cells analysed in this study,

with >95% of the hepatocyte population in the liver infected, it is expected that up to 800 – 8000 integrated virus-cell junctions would be detected, with hepatocyte clone sizes of at least 8 cells. Similar to results obtained in Chapter 7, the failure to detect more integrated DHBV-cell junctions in these studies could be due to high levels of virus-virus (false positive) sequences detected using the invPCR assay (81% of total invPCR products). It is of course possible that physiological differences between mammalian and avian liver biology could explain the lack of viral DNA integration and subsequent formation of hepatocyte clones. On top of that, as the timeframe of this study (327 dpi) was much shorter compared to 2.4 years post infection in woodchucks (Mason *et al.*, 2005) and at least 20 years post infection in humans and chimpanzees (Mason *et al.*, 2009a; Mason *et al.*, 2010), such large hepatocyte clones would not be expected to be found in ducks.

Declaration of originality and consent

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Huey Chi LOW 3rd April 2012

Publications and presentations resulting from this thesis

Manuscripts published

Mason, W., **Low, H.**, Xu, C., Aldrich, C., Scougall, C., Grosse, A., Clouston, A., Chavez, D., Litwin, S ., P eri, S ., J ilbert, A . and Lanford, R. (2009). D etection of c lonally expanded hepatocytes i n chimpanzees w ith c hronic h epatitis B vi rus i nfection. Journal of V irology. 83(17): 8396-8408.

Mason, W., Xu, C., **Low, H.**, Saputelli, J., Aldrich, C., Scougall, C., Grosse, A., Colonno, R., Litwin, S . a nd Jilbert, A. (2009). T he a mount of he patocyte t urnover t hat oc curred dur ing resolution of transient hepadnavirus infections was lower when virus replication was inhibited with Entecavir. Journal of Virology. 83(4): 1778-1789.

Manuscript in preparation

Reaiche, G., Thorpe, M., **Low, H.**, Qiao, Q., Scougall, C., Mason, W. and Jilbert, A. Evidence that cccDNA survives mitosis during antiviral therapy for chronic hepadnavirus infections.

GenBank publications

Low, H., R eaiche, G ., J ilbert, A. (2010) *Anas platyrhynchos* beta-actin g ene, p artial cd s. GenBank Accession GU564232.

Low, H., R eaiche, G ., J ilbert, A . (2010) *Anas platyrhynchos* glyceraldehyde-3-phosphate dehydrogenase-like protein (GAPDH) gene, partial cds. GenBank Accession GU564233.

Oral presentations

Low, H., B eard, M ., J ilbert, A . U sing i ntegrated he patitis B vi rus (HBV) D NA a s genetic marker. School of Molecular and Biomedical Science PhD Symposium, Adelaide, July 2009.

Thorpe, M., Reaiche, G., **Low, H.**, Mason, W., Jilbert, A. Evidence that the episomal DNA that serves as the transcriptional template of duck hepatitis B virus is able to survive mitosis in the growing liver. International Hepatitis B Meeting, San Diego, USA, August 2008.

Low, H., Beard, M., Jilbert, A. Using integrated hepatitis B virus DNA as genetic marker for individual i nfected he patocytes. T he U niversity of A delaide P hD m ajor r eview, A delaide, September 2008.

Low, H., Grosse, A., Scougall, A., Aldrich, C., Xu, C., Hall, P., Clouston, A., Lanford, R., Mason, W., Jilbert, A. Detection of clonally expanded hepatocytes in chimpanzees with chronic hepatitis B virus infection. Australian Centre for HIV & Hepatitis Virology Research (ACH^2) Annual Scientific Meeting, Barossa Valley, South Australia, June 2008.

Low, H., Mason, W., Jilbert, A. Using integrated hepatitis B virus DNA as genetic marker for individual infected hepatocytes. IMVS Infectious Diseases Laboratories (IDL) research seminar, Adelaide, July 2007.

Low, H., Mason, W., Jilbert, A. Using integrated hepatitis B virus DNA as a genetic marker of individual infected hepatocytes. The University of Adelaide Integrated Bridging Program (IBP) talk, Adelaide, June 2007.

Low, H., Mason, W., Jilbert, A. Using integrated duck hepatitis B virus DNA as a genetic marker for individual infected hepatocytes. Australian Centre for Hepatitis Virology (ACHV) Annual Workshop, Melbourne, May 2007.

Poster presentations

Low, H., Stroeher, U., Mason, W., Jilbert, A. Using hepatitis B virus-cell junctions as genetic markers for individual infected hepatocytes. Australia Virology Group (AVG) Meeting, Lorne, Victoria, December 2009.

Low, H., Stroeher, U., Mason, W., Jilbert, A. Using hepatitis B virus-cell junctions as genetic markers for individual infected hepatocytes. The University of Adelaide, School of Molecular & Biomedical Science School Research Symposium, Adelaide, December 2008.

Low, H., Mason, W., Jilbert, A. Using hepatitis B virus DNA as a genetic markers of individual infected hepatocytes. Australian Society for Microbiology (ASM) Annual Scientific Meeting, Adelaide, July 2007.

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Abbreviations

µ	micro
<	less than
~	approximately
^{o}C	degree Celsius
%	percent
1H.1	mouse anti-DHBV preS antibodies, IgG2a subtype
2’ CDG	2’ carbodeoxyguanosine
A	adenine
A	Ampere
AB	Applied Biosystems, now part of Life Technologies
AEC	Animal Ethics Committee
AGRF	Australian Genome Research Facility
ALT	alanine aminotransferase
AR	analytical reagent
AST	aspartate-aminotransferase
AU	Anson unit
AusDHBV	Australian strain of DHBV, Genbank Accession AJ006350
b	base(s)
BD	Becton Dickinson
BLAST	Basic Local Alignment Search Tool
bp	base pair(s)
BrdU	Bromodeoxyuridine
BSA	bovine serum albumin
c	centi
C	cytosine
C	core
$CaCl_2$	calcium chloride
cccDNA	covalently closed circular DNA
CDC	Centers for Disease Control and Prevention
cDNA	complementary DNA
Ci	curie
CMI	cell mediated immunity
CPM	counts per minute
CSU	Central Services Unit, the University of Adelaide, SA

CTL	cytotoxic T-lymphocyte
DAB	diaminobenzidine tetrahydrochloride
DAPI	4',6-diamidino-2-phenylindole
ddNTP	dideoxy nucleotide tri-phosphate
DHBcAg	duck hepatitis B core antigen
DHBsAg	duck hepatitis B surface antigen
DHBV	duck hepatitis B virus
DMSO	dimethyl sulfoxide
DNA	deoxyribo nucleic acid
dNTP	deoxy nucleotide tri-phosphate
DOP PCR	semi-degenerate oligonucleotide-primed PCR
dpi	day(s) post infection
DR	direct repeat
dslDNA	double stranded linear DNA
dUTP	deoxyuridine tri-phosphate
EAA	ethanol:acetic acid (3:1 v/v)
EB	elution buffer
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
EMBO	European Molecular Biology Organization
E. coli	*Esterichia coli*
ER	endoplasmic reticulum
ETV	entecavir
FCCC	Fox Chase Cancer Center, Philadelphia, USA
FCS	foetal calf serum
FTC	emtricitabine
g	gram(s)
g OR x g	x gravitational force, or known as RCF
G	guanine
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GE	genome equivalents
GE	General Electric Company, Sweden
GSHV	ground squirrel hepatitis virus
H&E	haematoxylin and eosin
H_2O	water
H_2O_2	hydrogen peroxide

H_2SO_4	sulphuric acid
HAART	highly active antiretroviral therapy
HBcAg	HBV core antigen
HBeAg	hepatitis B e surface antigen
HBsAg	HBV surface antigen
HBV	hepatitis B virus
HBxAg	heptatitis B X protein
HCC	hepatocellular carcinoma
HCl	hydrochloric acid
HCV	hepatitis C virus
HI	digested at high concentrations
HIV	human immunodeficiency virus
HNF	hepatocyte nuclear factor
HPRT1	hypoxanthine-guanine phosphoribosyl transferase I
hr	hour(s)
HRP	horseradish peroxidase
IFN	interferon
IgY	immunoglobulin Y
IL	interleukin
IMVS	Institute of Medical and Veterinary Science, SA Pathology
invPCR	inverse nested PCR
IU	International Unit
k	kilo
kb	kilo base pairs
KCl	potassium chloride
L	litre(s)
LB	Luria broth
LHB	large hepatitis B surface proteins
LNA	locked nucleic acid
LO	digested at low concentrations
m	metre(s)
m	mili
M	molar(s)
M	mega
MCS	multiple cloning site

$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphite
MHB	middle hepatitis B surface protein
MHC	major histocompatibility complex
mIU	mili International Unit(s)
min	minute(s)
mol	mole
mRNA	messenger RNA
MW	molecular weight
n	nano
N/A	not available
NA	nucleoside/nucleotide analogue(s)
Na_3Citrate	trisodium citrate
NaCl	sodium chloride
Na_2HPO_4	disodium hydrogen phosphate
NaH_2PO_4	monosodium phosphate
NaN_3	sodium azide
NaOAc	sodium acetate
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
ND	not detected
NDS	normal duck serum
NEB	New England Biolabs, USA
NHMRC	National Health and Medical Research Council, Australia
NK	natural killer
no.	number
NP40	nonyl phenoxylpolyethoxylethanol Tergitol-type NP-40
NSS	normal sheep serum
NSW	New South Wales, Australia
nt	nucleotide(s)
OD	optical density
OH&S	Occupational Health and Safety
OPD	o-phenylenedianine dihydrochloride
ORF	open reading frames
p	pica
P	polymerase

PAMPs	pathogen-associated molecular patterns
PAS-D	periodic acid-Schiff diastase
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline- tween
pBsSK	pBluescript SK II - plasmid
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
pfu	plaque forming units
PLoS	Public Library of Science
PNAS	Proceedings of the National Academy of Sciences of USA
PVP	polyvinylpyrrolidone
qPCR	quantitative PCR
rcDNA	relaxed circular DNA
RCF	Relative Centrifugal Force
RI	replicative intermediates
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	reverse transcription
RT	room temperature
S	surface
SA	South Australia, Australia
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulphate
sec	second(s)
SHB	small hepatitis B surface protein
SOX5	Or1 sex determining region Y-box 5
T	thymine
TAE	tris acetate EDTA
TBP	TATA-binding protein
TE	tris EDTA
TFV	tenofovir
Th1	type 1 T helper cells
Th2	type 2 T helper cells
TLR	Toll-like receptors

Tm	melting temperature
TNF	tumour necrosis factor
TNV	tenofovir
Tris HCl	tris(hydroxymethyl)aminomethane
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
U	unit(s)
ULN	upper limit of the normal range
USA	the United States of America
UV	ultraviolet
V	Volt(s)
vge	virus genome equivalent
WHO	World Health Organization
WHV	woodchuck hepatitis virus
x	time(s)
YY1	Yin and Yang 1