FINAL REPORT 27th May 2010

BIOTECHNOLOGY AND BIOLOGICAL SCIENCES RESEARCH COUNCIL

RESEARCH GRANT INTERIM REPORT FORM

PROJECT DETAILS

Grant number

BB/D012171/1

Award holding organisation

Organisation	University of Warwick	Research Organisation Reference:	
Division or Department	Department of Biological Sciences		

Title of research project

The application of reverse genetics to the study of pathogenicity in avian pneumovirus

Project details

Total grant value (£)	440,694.69
Start date	1/1/06
End date	5/4/10
Original duration of grant (months)	48
Extension (as agreed with BBSRC Office)	

Investigators

Role	**Name**	**Organisation**	**Division or Department**
Principal investigator	Professor A. J. Easton	**University of Warwick**	Biological Sciences
Researcher co-investigator			
Co-investigator	Dr C. J. Naylor	University of Liverpool	Veterinary Pathology

Grant number

BB/D012171/1

Objectives - the main objectives of the research in order of priority (as in original research proposal)

The proposal has four objectives.

1. To determine whether differences in pathogenicity between wild type and cell culture adapted APV are primarily due to mutations or differences in modifications occurring in cell cultures and the natural host.
2. To investigate the hypothesis that vaccine strains of APV replicate in a different target tissue from virulent strains.
3. To determine whether cell-cell fusion of APV infected cells is inhibited by the viral SH protein or if the SH protein is required for the normal non-syncytial phenotype that is lost when the SH gene is deleted. To investigate the role of the two domains of the SH protein in altering the cytopathic phenotype.
4. To determine the pathogenicity and protective capabilities of viruses with deleted genes.

Changed objectives (as agreed with BBSRC Office)

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Technical summary (as in original application)

Avian pneumovirus (APV) causes respiratory disease of economic importance to the poultry industry worldwide. Vaccines have been produced but are prone to reversion to virulence. The genetic basis of attenuation of APV is not understood. Our unpublished data shows that genome mutations, alterations in post-translational modifications or a combination of these may be involved. Virus is often attenuated by culture in mammalian cells which can be associated with differences in glycosylation of at least one of the viral surface glycoproteins. It is hypothesized that the site of replication of the virus may be restricted in attenuated viruses and that this enables the birds to develop a protective immune response before disease can develop. The two laboratories have identified a number of mutations associated with attenuation in different genetic backgrounds of APV and these are located in a restricted number of virus genes. Some alterations have been identified in the virus intergenic regions which may have an effect on virus gene expression.

Using a reverse genetics system for APV developed collaboratively by the two applicant laboratories (Naylor et al., 2005, J Gen Virol, 85, 3219-3227) we will identify the mutations responsible for the attenuated phenotype in APV. Using the reverse genetics system specific, site-directed, mutations will be introduced into the virus genome either by PCR based methods or QuikChange mutagenesis (Stratagene) on subclones followed by cloning into a full-length clone we have modified to contain useful restriction sites. We will introduce singly and together a range of specific mutations into the APV genome from two different isolates and assess the effects on pathogenicity in birds. Using a dicistronic minigenome system (Randhawa et al., 1997, J Virol 71, 9849-9854) we will assess the impact of the intergenic mutations on expression of a downstream gene by measuring the relative expression levels of two reporter genes. Northern blot analysis and ribonuclease protection studies will enable measurements to be made on expression of actual genes in mutant viruses.

The reverse genetics system also allows the insertion or deletion of genes. The green fluorescent protein ORF bounded by appropriate viral regulatory sequences will be introduced into full-length viral clones using assembly PCR. This virus will be used to follow the spread of virus in infected birds with wild type and mutant genomes. This will clarify the nature of any tropism restriction that exists with the viruses. This work will be carried out using virus propagated in different cell lines to assess the importance of host-derived factors in the initial stages of infection in vivo. In addition, we investigate the function of the SH protein in cell-cell spread of virus. In contrast to other viruses deletion of the SH gene alone in APV causes a phenotypic change with enhanced cell-cell fusion being produced in cell culture. Studies in cell culture on the importance of two apparent domains in the APV SH protein on its function will be carried out using viruses modified as above with alterations to conserved residues or systematic replacement of amino acids, the syncytial phenotype being used as a functional screen. The way in which this phenotype is established, either by loss of a fusion inhibiting function or of a function required for development of a non-syncytial phenotype, will be studied by induction of SH expression at different times after infection by SH deleted virus. Suitable cell lines will be established expressing functional or non-functional SH protein expressed in an inducible manner from the same locus in the chromosomal DNA using the tet Flp-In system (Invitrogen). This will provide information on the regulation of fusion activity and the function of the SH protein. The sub-cellular location of SH protein will be identified by immunofluorescence using virus expressing a tagged SH protein.

Summary report – a summary of progress on the grant (up to 2 A4 pages)

Control of gene expression in AMPV

Analysis of mRNA levels in tissue culture showed that the transcription gradient for AMPV is remarkably shallow with all genes transcribed to similar levels except for the L mRNA which was expressed approximately 10-fold less than the others. The non-consensus GS sequence for the AMPV L gene may account for this difference. Limited studies on the transcription pattern of AMPV in turkeys using real time PCR determined that the situation in vivo is similar to that observed in cell culture. In contrast, using a method to compare protein levels it was seen that the amount of the proteins varied far more than had been observed for the RNA levels in other AMPVs with approximately five fold differences in the order P>F>SH. These data raise questions about current models of metapneumovirus gene expression.

Mutations affecting pathogenicity

A virulent revertant virus (isolate 309/04) was form in a Vero cell-attenuated live vaccine some weeks after vaccination. Complete genome sequencing showed this to carry 5 mutations compared to the parent vaccine virus: 2 were just prior to the F gene and 3 were within the polymerase (L) gene. Only 2 in the polymerase gene generated coding changes. Recombinant viruses were generated from the vaccine and revertant isolates. Both behaved as the parental viruses demonstrating that the phenotypic changes were the result of genetic changes. Further mutational analysis showed that a single coding mutation in the L gene was responsible for the increased virulence in turkeys. This virus had similar growth characteristics and gene expression patterns to the attenuated parental virus at 37C but was significantly more stable at 40C implicating this as a key factor. Replacement of the F gene from a virulent virus into a virulent genetic background had little effect on virulence showing that the F gene is not the sole virulence determinant. However the modification did convert the previously non-protective virus into one inducing a very high level of protective immunity. Deletion of either SH or G genes from this modified virus led to equivalent reductions in virulence as well as moderate decrease in induced protection against virulent challenge. However SH gene deletion also led to a dramatic reduction in post inoculation virus shed and may indicate that this virus replicates in a tissue beyond the respiratory tract.

The role of the SH and F proteins in fusion activity

Tissue culture cells transiently expressing the AMPV F protein produce syncytia, demonstrating that the F protein alone has this capacity. Protein localisation studies showed that the SH protein interacted with the F protein but that there was no co localization between either SH or F proteins with lipid rafts.

SH gene mutants

AMPV mutants were created with either premature stop codons or delayed transcription and translation starts in the SH gene to shorten the protein at either the N or C terminus. All constructs produced a giant syncytial phenotype in cell culture with the exception of the virus with a premature stop at nucleotide residue 238. This implies that only the amino terminal part of the SH protein is needed for "normal" CPE. However expressing more of the protein leads to giant syncytial CPE but once the full protein is made CPE returns to "normal".

Sequence analysis identified a potential second ORF in the SH gene of all AMPV strains which has not previously been studied. We have demonstrated that this ORF encodes a protein by insertion of GFP into the SH gene in frame with the previously unrecognised ORF. No clear phenotype has been identified with mutation of this ORF. Site-specific mutagenesis of the SH gene produced three mutants, with syncytial plaques indicating a loss of function associated with the mutations. All three affected residues are conserved in all AMPV subtypes. This is the first identification of critical residues necessary for the SH protein role in syncytia formation.