Ectromelia virus lacking the E3 gene is replication-defective

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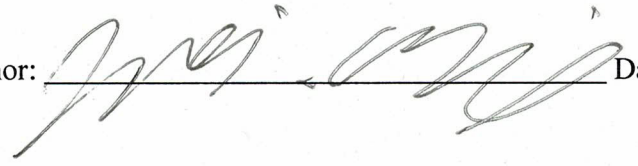
Signature of Author:  Date: 4/11/2019

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Abstract

Many orthopoxviruses, including ECTV, contain a common gene, E3L. This gene produces a protein, E3, that binds to viral double stranded RNA and helps evade host immune pathways, such as PKR. Using a mutant virus with an interrupted E3L gene (ECTV) we found that this mutant had a halted replication cycle that resulted in no translation of any late genes. This replication was rescued in cultures where E3L was added to cells or where PKR was knocked out.

Introduction

Poxviruses are a family of viruses that have large, brick or ovoid shaped virions, and a double stranded DNA genome[1]. Many of these viruses have evolved over time to have different functions which allow them to infect and survive in their hosts[1]. From poxviruses comes the genus of orthopoxviruses, which is of particular interest to this study because they include ectromelia (ECTV), or mousepox, which is the main type of virus looked at for this study [2]. Other interesting viruses in this family are vaccina (VACV), variola, monkeypox, and cowpox virus[2].

Variola virus (VARV) is the causative agent of smallpox which was one of the most serious and fatal diseases humans have encountered[1,2,3]. It was endemic to every country, except Australia and some islands[1]. It was able to spread so efficiently because it was most commonly transmitted through respiratory droplets between human hosts[2]. It had a fatality rate of up to 40%, and as a result it killed thousands of people[1,2]. Luckily VARV has a very limited host range and no natural animal host, so it was able to be eradicated naturally from the world by an effort by the WHO in the 1970s[1,3]. While VARV is now eradicated in nature it can still be found in vaults at the CDC in Atlanta, Georgia and the Vector Institute in Russia, and some have begun to fear that terrorist groups might also have it and potentially weaponize it[3]. This is the primary reason why orthopoxviruses are currently being given so much attention, so that researchers will be ready to act in the case of bioterrorism[3].

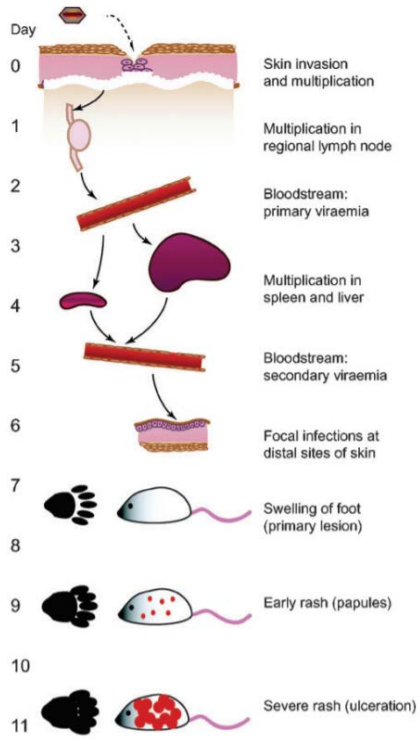


Figure 1. Infection of ECTV using the footpad method (Esteban and Buller 2005).

ECTV is a good virus to use as a model for VARV infection for a variety of reasons, one of which is that it also has a very specific host range in that it can only infect mice[1]. It also acts clinically very similarly to that of smallpox[3]. It is most commonly transmitted to the mice through their footpads, the virus can enter through the epidermis and dermis to infect the rest of the mouse (Figure 1)[3]. When infecting mice in a laboratory setting, the virus is injected into the footpad of the mice to mimic natural infection, called the footpad model[1,3]. After initial infection the virus travels to the closest lymph node, typically in the knee called the draining lymph node[3]. From here the virus can travel into the blood and spread the infection, causing primary viremia[1]. Then the virus travels to the spleen and liver where the virus infects the tissues and results in necrosis and dispersal of the infected tissue directly into the blood, causing secondary viremia[1]. Eventually the virus reaches the skin and replicates rapidly, causing the characteristic pox lesions typically seen during poxvirus infections[1]. At this point the mouse is at its most infectious point and can begin to infect other mice around it[1].

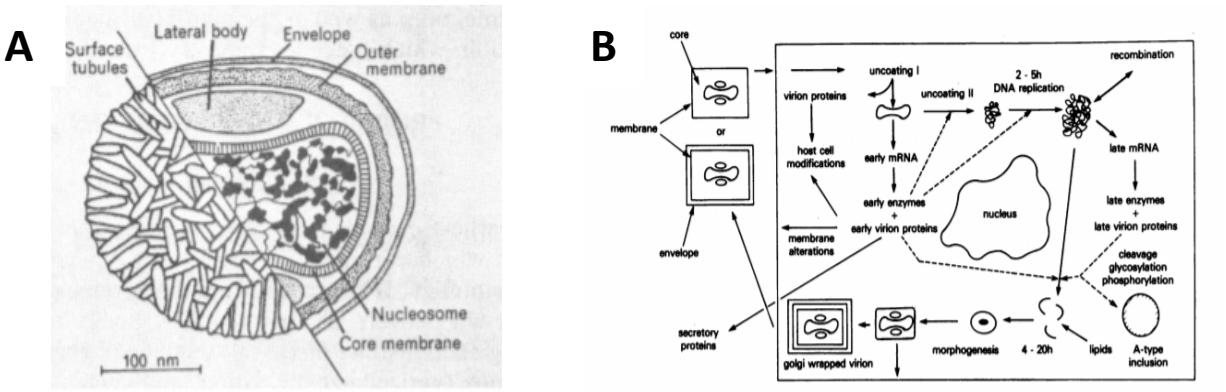


Figure 2. Structure and life cycle of ECTV. A) Detailed structure of an ECTV virion (Buller and Palumbo 1991). B) Lifecycle of a poxvirus after it enters a host cell (Buller and Palumbo 1991).

At a cellular level ECTV and all the other orthopoxviruses cannot spread the infection to new cells, tissues, organs, and other organisms without replication of its virions in the cytoplasm of the infected host cells[1]. The virus enters the host cell through a process of uncoating which in ECTV removes its outer membrane which holds the lateral bodies and a core that contains the virus's DNA (Figure 2)[1]. Once the virus enters the cell it immediately begins transcription of the early viral genes that are responsible for host interactions[1]. Then begins replication of the viral DNA to begin the process of making more virions that will eventually infect other cells[1]. This all occurs in the cytoplasm of the host cell because ECTV provides the transcription components without the use of host machinery[1]. Then begins late gene transcription. These genes are involved with assembly of the virion so they are packaged and ready to leave the cell[1]. Lastly is the final assembly of the virion and virus dissemination where the complete virions are now able to travel to the host cell membrane where they are exocytosed through exosomes and infect other cells[1].

ECTV is a better model of study to develop a vaccine for VARV than those based on vaccinia virus (VACV)[4]. VACV is another orthopoxvirus whose antigens were used as the vaccine for VARV to help eradicate the virus back in the 1970s[4]. It was an extremely effective

vaccine against VARV back when it was an obvious threat to humanity, but now that it has been eradicated from nature and there is only the threat of bioterrorism that would require nations to reintroduce a VARV vaccine a safer vaccine is needed[4]. The VACV vaccine was effective but also had very severe side effects such as encephalitis, eczema, and heart complications which would be much harder on the increased population of immunocompromised people due to AIDS, organ transplants, and cancer treatments[4].

Currently attenuated strains of VACV are being looked at, so patients would be able to still get a live virus vaccine but would have little to no side effects[4]. That is the ideal vaccine vector, but it is difficult because researchers look at the host-virus interactions in mice, which VACV cannot infect[4,5]. As mentioned previously ECTV is a good model for VARV because it also has a specific host of mice instead of humans[1]. This makes it a good model because the host-virus interactions would be natural and safer due to the host restrictions of ECTV, and that mice are a common laboratory animal used, so it's also a better virus to use for convivence's sake[6]. ECTV also has similar genes as VARV, including the E3L gene, of which they also share with VACV[5].

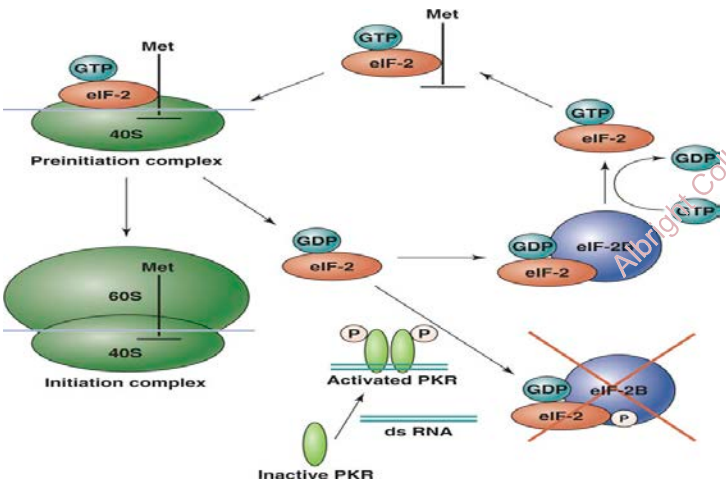


Figure 3. Viral dsRNA: PKR pathway (Acheson 2011).

One potential gene that has been most targeted to make an attenuated vaccine is the VACV E3L gene, which codes for an inhibitor of protein kinase R (PKR) by stopping dsRNA induced PKR activation[5,7,8]. This gene is also shared by other members of the orthopoxvirus family, such as VACV[13]. An important part of ECTV

virion replication is dsRNA produced during the process[9,10]. It is a major pathogen associated molecule (PAMP) that hosts use to detect viral infection and initiate an anti-viral assault[10]. Typical RNA during replication is single stranded and when double stranded RNA is detected that is a major indication that the cell is infected by a virus[10]. This dsRNA is a hallmark of viral infection because the virus has a lot of genes in a small amount of space so during transcription there can be mRNAs that have overlapping sequences due to the viral RNA polymerase not terminating effectively[10]. The E3L gene and its protein product E3 are so important to evading the host's immune system because the PKR pathway inhibits viral translation and replication, but E3 helps to halt this[10,11,14]. When the PKR pathway detects viral dsRNA it phosphorylates, and therefore inhibits, the alpha subunit of eukaryotic initiation factor 2 (eIF2α) which then cannot bring the amino acid methionine to start translation in the ribosome, halting translation and protein synthesis so virion replication is terminated (Figure 3)[10,11]. This pathway is avoided through the E3L gene product E3 being able to sequester the viral dsRNA so that PKR is not able to find and inhibit it[11,15].

Many discoveries about the functions of viral genes and have been accomplished through knockout experiments[8]. Knockout viruses contain a disrupted specific viral that produces a change in the protein product that results in a phenotypic change to the viral infection[8]. An important aspect to this system is that an animal model is required, becasue it is easier to see how the virus is effecting the host. Since ECTV naturally infects mice it is a good virus to use in performing a knockout of a gene, like E3L, to determine the importance of its function[8]. In this study we use this technique to better understand the importance of this gene to the replication of ECTV.

Materials and Methods

Cell Culture and Cells

For this study we used these cell lines: BS-C-1, HeLa, L-929 and MEF. BS-C-1 are a cell line derived from monkey kidney cells, HeLa cells are human ovarian cancer cells, L-929 are mouse connective tissue cells, and MEF are mouse embryonic fibroblast cells. All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (FBS), and penicillin-streptomycin. All the cells were kept at 37°C in a 6% CO_2 incubator and split weekly or as needed.

Viruses

For this study we used these viruses: ECTV wild-type (Moscow strain), ECTV expressing GFP (Moscow background), and ECTVΔE3L. ECTVΔE3L was made using was homologous recombination techniques and expresses green fluorescent protein (GFP). The E3L open reading frame had 90% replaced with GFP and a viral promoter was used to ensure it was being transcribed (Figure 4). After infecting/transfecting with ECTVΔE3L, GFP-positive plaques were isolated and passaged six times in BS-C-1+E3L cells to make sure that all the viral plaques expressed GFP so we knew we had the virus we wanted. We also made a revertant version of ECTVΔE3L (ECTV E3L REV) that had E3L reintroduced back into ECTVΔE3L. After this we underwent the same process that we used to get ECTVΔE3L to make sure we had isolated our revertant virus.

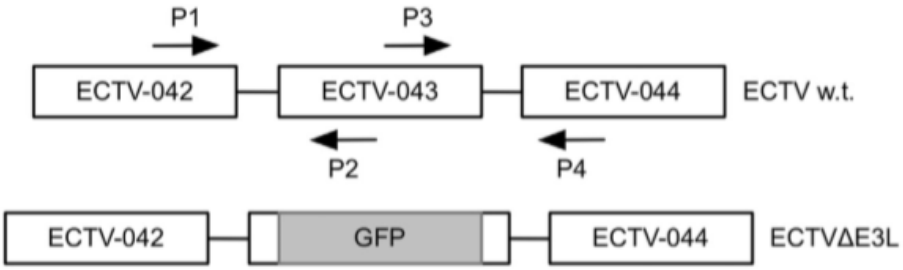


Figure 4. Drawing of the genome of w.t. ECTV in the region of the E3L gene and the location where GFP was inserted to disrupt E3L. The arrows show where PCR primers bound (Frey et al, 2018).

Fluorescence Microscopy

This was done using a Zeiss Axiostar plus epifluorescence microscope, and the images were taken with an Optronics camera system. All the images were altered (brightness, contrast, etc.) using ImageJ software. To get the pictures we grew the cells in four-well chamber slides and infected with w.t. ECTV or ECTVΔE3L, and then incubated in 5% formalin for 10min at room temperature. Then for surface B5 staining, unpermeabilized cells were fixed and then incubated with an anti-B5 monoclonal antibody. For actin, the cells were fixed and permeabilized and incubated in the presence of Alexa Fluor 488phalloidin. All slides had a glass coverslip mounted using ProLong Gold antifade reagent with 4'-6-diamidino-2-phenylindole (DAPI).

Flow Cytometry

A minimum of 75,000 total cells were collected for each sample, and the data was analyzed using FCS Express 4 Flow Cytometry. To determine the spread of virus, BS-C-1 w.t. or BS-C-1+E3L cells were seeded into six-well plates and then were infected with GFP-ECTV or ECTVΔE3L. After 24, 48, 72, and 96h of infection, the cells were harvested using trypsin and immediately analyzed using a FACSCalibur instrument that had red and blue lasers.. Viral

spread in culture was quantified by measuring the percentage of total cells that were positive for GFP expression at each time point.

Reporter Plasmid

To determine late gene expression, a plasmid was constructed using the GeneArt Synthesis service that had the gene for mCherry. The promoter for the VACV gene A14L, a known late gene, was used and placed upstream of the start codon for mCherry. The promoter for the ECTV A14L ortholog differs by a single nucleotide from the VACV sequence, so it worked for ECTV and would promote mCherry as a late viral gene. To determine the amount of mCherry produced, BS-C-1 cells were transfected and infected with the indicated viruses. The reporter gene plasmid was introduced into cells using the Lipofectamine 3000 kit, and fluorescence was measured using microscopy at the indicated time points post-infection.

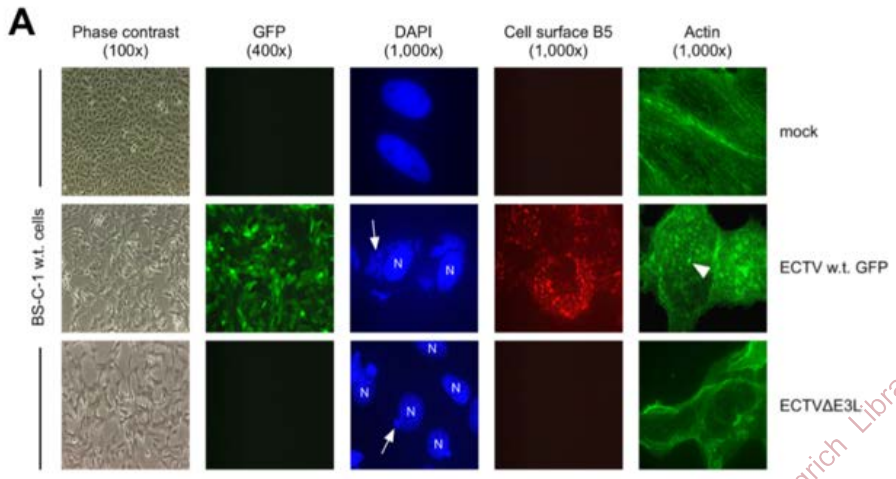
Western Blotting

Cells were plated in 6-well culture plates, and then infecting or mock treating them the following day. Infections were carried out in DMEM+2% FBS for one hour in minimal volume followed by replacement with standard media. To detect eIF2α in the plates, protein was collected at 6h post-infection using cell extraction buffer with Halt™ Protease and Phosphatase Inhibitor Cocktail and EDTA. A total of 100µg of protein for each sample was separated using 12% Mini-PROTEAN TGX gels, and then transferred onto a 0.45µm polyvinyl difluoride (PVDF) membrane. Membranes were blocked using StartingBlock™ T20 in TBS blocking buffer. To detect A3 protein (a late gene), cells were infected for 18h prior to isolating total protein as described previously. For protein detection, the primary antibodies were used: rabbit anti-phospho eIF2α, mouse anti-total eIF2α, mouse anti-beta actin, and rabbit anti-VACV A3.

Detection using the corresponding secondary antibody conjugated to alkaline phosphatase was used for each blot.

Results

To compare what infection looked like in BS-C-1 cells with w.t. ECTV or ECTVΔE3L using a phase contrast microscope, fluorescence microscopy, and DAPI, actin, and cell surface B5 stains. While analyzing the results we found that the BS-C-1 cells infected with ECTVΔE3L had an abortive effect on viral replication (Figure 5A). Viral factories were smaller in the w.t. BS-C-1 cells infected with ECTVΔE3L, and there was no surface B5 expression (a late gene). There was actin, but no actin tails, which are a late viral phenomenon. When BS-C-1 cells were infected in conjunction with E3L, replication is restored in ECTVΔE3L so that infection is like that of w.t. ECTV (Figure 5B).



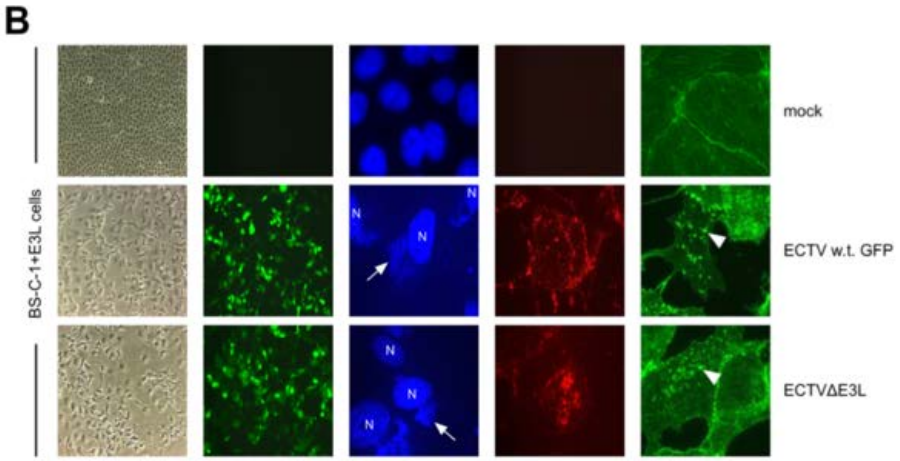


Figure 5. Infection of w.t. BS-C-1 cells with ECTVΔE3L resulted in an abortive replication cycle of the virus. A) w.t. BS-C-1 B) BS-C-1+E3L cells infected with w.t. ECTV and ECTVΔE3L. After 24 hrs. post infection cells were examined for GFP, viral factories (indicated to with arrowheads) and cell nuclei stained with DAPI for cell nuclei, B5 at the cell membranes, and actin tails (indicated with arrow heads).

We next looked to see if the PKR pathway is activated during ECTVΔE3L infection. Total protein was extracted from BS-C-1, HeLa, and L-929 cells infected with w.t. ECTV or ECTVΔE3L. A Western blot was done on the protein to detect phosphorylated eIF2α, total eIF2α, and actin (as a loading control). After analysis it was found that there was no phosphorylated eIF2α protein in the w.t. ECTV infected cells, but there was in the cells infected with ECTVΔE3L (Figure 6). There were similar levels of total eIF2α and actin so loading was consistent.

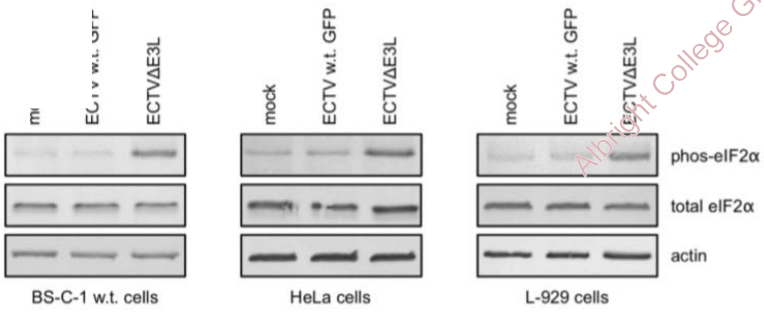
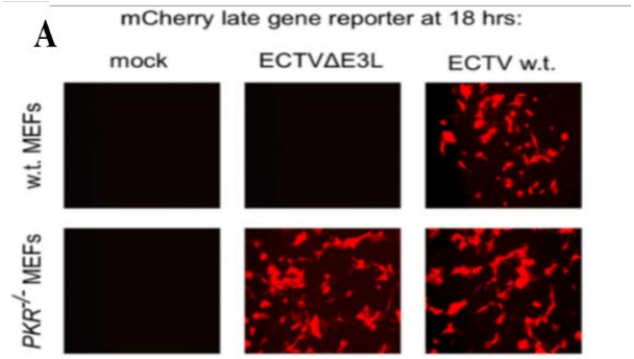


Figure 6. The anti-viral pathway that inhibits the replication cycle of ECTVΔE3L is PKR. W.t. BS-C-1, HeLa, and L-929 cells were infected with w.t. ECTV and ECTVΔE3L, and then after 6 hrs. the total protein was isolated. A Western blot was done on the protein to detect phosphorylated eIF2α, total eIF2α, and actin.

Lastly, late gene transcription was looked at to determine how far into the replication cycle ECTVΔE3L could go before being terminated by the PKR pathway. PKR double knockout MEFs was used to see if ECTVΔE3L transcription could be restored in a line with no PKR present. A mCherry reporter plasmid with a late gene promoter was transfected/infected into the cells, and then after 18 hrs, was visualized using fluorescence microscopy (Figure 7A). In the w.t. MEFs infected with ECTVΔE3L there was no fluorescence, but it was detected in the cells infected with w.t. ECTV. In the MEFs $PKR^{-/-}$ there was fluorescence in both conditions with virus added. Analysis reveals that when PKR is knocked out transcription of late genes is restored. This same effect is seen 24 hrs. post infection with the viruses and then visualized for GFP, virus factories and cell nuclei, and cell surface B5 (Figure 7B). In the MEFs $PKR^{-/-}$ cells infected with ECTVΔE3L there is GFP expression, the viral factories are more comparable to those of w.t. ECTV, and there is surface B5 production. This is not seen in the w.t. MEFs where there the PKR present terminated transcription of ECTVΔE3L. A Western blot was also used to detect the late viral gene, A3, in conditions with and without AraC (Figure 7C). AraC is an inhibitor of viruses and so when it was added to w.t. ECTV there was no A3 detection because it was not being translated, and when it wasn't added along with w.t. ECTV infection A3 was detected. With ECTVΔE3L there was no A3 bands on the blot, like that of the AraC added w.t. ECTV lane, meaning the late gene was not being translated.



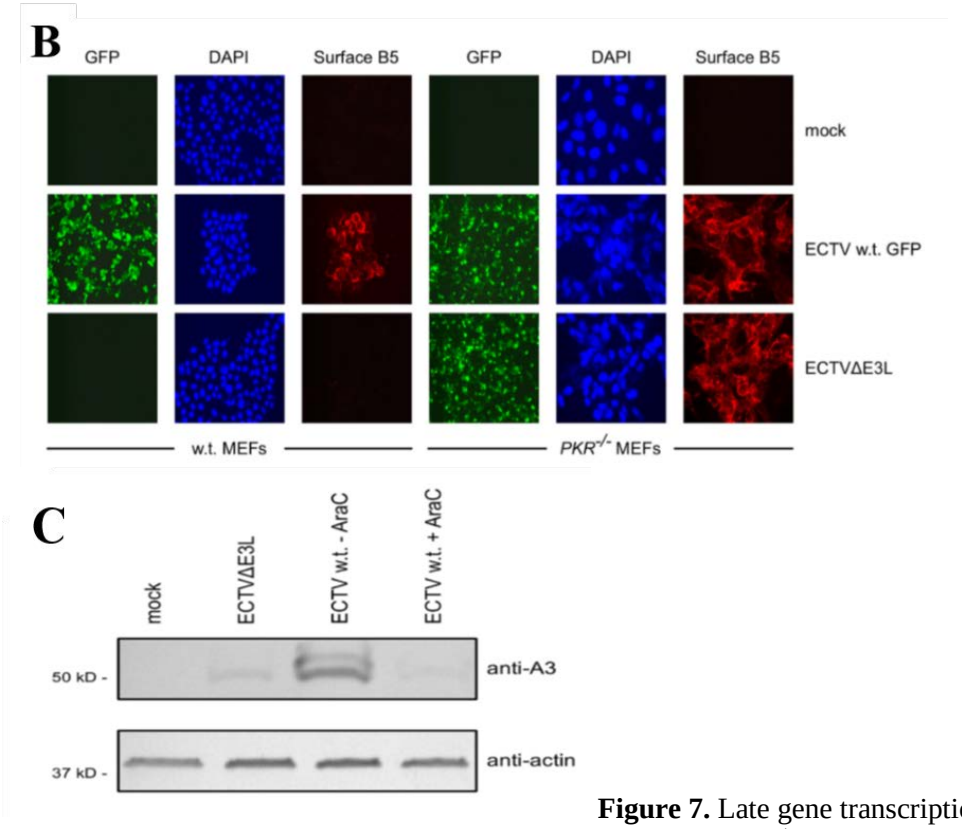


Figure 7. Late gene transcription in ECTVΔE3L was detected but their translation was blunted. A) w.t. MEFs and PKR$^{-/-}$ MEFs were transfected/infected with the mCherry plasmid and then w.t. ECTV and ECTVΔE3L was added, and then after 18 hrs. was visualized using fluorescence. B) w.t. MEFs and PKR$^{-/-}$ MEFs were infected with w.t. ECTV and ECTVΔE3L, and after 24 hrs. the cells were visualized for GFP, virus factories and cell nuclei, and cell surface B5. C) w.t. BS-C-1 cells were infected for 18 hrs. and total protein was isolated. A Western blot was done to detect A3, and actin as a loading control. AraC was added at time of infection.

Discussion

In this study we have demonstrated that ectromelia virus is replication defective when the E3L immune evasion gene is lost. The E3L gene is responsible for ECTV's ability to evade the PKR pathway using its protein product E3 to sequester the dsRNA that ECTV makes during transcription so that the virus can complete replication[10,11].

Our results help to back this information by showing that without the E3L gene the virus is unable to complete transcription and has an abortive effect that results in smaller virus

factories, no surface B5, no actin tails, and no GFP expression. When E3L is added back in the BS-C-1 cells transcription is rescued and there is little difference from the w.t. ECTV infection. This demonstrates how vital the E3L gene is to replication of the virus, and without it there is no transcription of late genes needed for final virion assembly[1,2].

We were able to determine that it was activation of the PKR pathway that was being activated by ECTVΔE3L using a Western blot analysis of infected cells to determine if there was any phosphorylated eIF2α. This would be indicative of the PKR pathway because when PKR is activated it phosphorylates eIF2α to stop translation, so its presence would show that PKR is being activated[10]. This was seen in our Western blots, so this shows that it is the PKR pathway being activated during ECTVΔE3L infection.

Lastly, we found that late ECTVΔE3L genes were not being translated, likely due to this PKR pathway activation. The reporter plasmid mCherry that was transfected/infected into MEF cells was under control of a late promoter, so its expression indicated late gene transcription. It was only seen in w.t. ECTV infected cells, and when ECTVΔE3L was added to $PKR^{-/-}$ MEFs. This showed us that loss of PKR was sufficient for late gene expression. Similar findings were seen when detecting surface B5, also a late gene. Another late gene, A3, was measured using a Western blot, and there was no expression for ECTVΔE3L again further giving evidence that when ECTV has its E3L gene removed late gene translation is terminated.

The ECTVΔE3L virus was created to better help us understand the role of the ECTV E3L gene and how vital a role it plays in virion replication. From the experiments that we did in this study we can make the connection that some intermediate and/or late protein products such as E3L might be needed for the completion of viral genome replication. This was expanded on *in vivo* by our collaborators, to better look at how this mutant virus affects mice compared to w.t.

ECTV. We do not have a mouse lab so while we worked with the virus on a cellular level, they looked more at host-virus interactions. It was seen that that this mutant virus after infection can offer protection from w.t. ECTV in mice where ECTV is typically fatal[11]. The next step after this study is to determine if ECTVΔE3L could be a possible vaccine vector.

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