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Cascade regulation of vaccinia virus gene expression is modulated by multistage promoters

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ABSTRACT

Vaccinia virus contains ~200 genes classified temporally as early, intermediate or late. We analyzed 53 intermediate promoters to determine whether any have dual late promoter activity. Our strategy involved (i) construction of a cell line that stably expressed the three late transcription factors, (ii) infection with a vaccinia virus mutant that expresses RNA polymerase but neither intermediate nor late transcription factors, and (iii) transfection with plasmids containing a luciferase reporter regulated by an intermediate promoter. After confirming the specificity of the system for late promoters, we found that many intermediate promoters had late promoter activity, the strength of which correlated with a TAAAT at the initiator site and T-content from positions −12 to −8 of the coding strand. In contrast, intermediate promoter activity correlated with the A-content from positions −22 to −14. The sequence correlations were confirmed by altering the specificities of strict intermediate and late promoters.
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Introduction

Poxviruses comprise a large family of DNA viruses that are noted for their medical importance, usefulness as vaccine vectors and ability to replicate entirely in the cytoplasm (Moss, 2013). Replication outside of the nucleus is attributed in large part to poxvirus-encoded proteins for genome replication and transcription. Gene expression is temporally programmed to occur in three consecutive stages: early, intermediate and late (Baldick and Moss, 1993). Transcription is mediated by a multisubunit DNA-dependent RNA polymerase and stage-specific transcription factors that recognize cognate promoters (Moss, 2013). Although characterized primarily for vaccinia virus (VACV), the transcription system is conserved in all chordopoxviruses. Early genes are transcribed with the aid of proteins that are synthesized late in infection and packaged within infectious virus particles (Broyles and Fesler, 1990; Broyles et al., 1988; Gershon and Moss, 1990). Intermediate and late genes are transcribed following genome replication and require the successive synthesis of intermediate and late transcription factors, encoded by early and intermediate genes, respectively (Keck et al., 1990; Sanz and Moss, 1999). Roles for specific cellular proteins in intermediate and late gene expression have also been reported (Katsafanas and Moss, 2004; Knutson et al., 2006, 2009; Rosales et al., 1994; Wright et al., 2001).

VACV genes that are expressed early are readily recognized by their transcription in the presence of inhibitors of protein and DNA synthesis. Based on these criteria, we classified the VACV transcripts for 118 open reading frames (ORFs) as early and 93 as post-replicative (Yang et al., 2010). Discriminating between intermediate post-replicative and late post-replicative transcripts is difficult because of their nearly concurrent synthesis and read-through of neighboring genes. The most satisfactory method has been to infect cells with VACV in the presence of an inhibitor of DNA replication, transfect candidate intermediate and late genes, and monitor protein synthesis. Only the intermediate genes are expressed because their transcription factors are products of early genes whereas late transcription factors are not made in the absence of DNA replication. Based on this criterion, we identified 53 intermediate and 38 late genes (Yang et al., 2011b). However, for technical reasons gene designations have been based on a triage system. Thus, genes have been designated early even though they might also be expressed at intermediate or late stages. Similarly, genes have been designated intermediate even if they might also be expressed late. Indeed, analyses of individual genes have indicated the existence of tandem early and post-replicative promoters (Cochran et al., 1985; Rosel et al., 1986; Vos and Stunnenberg, 1988) and others have been artificially constructed (Baldick and Moss, 1993). Identifying multistage promoters may help to better understand poxvirus replication and their use may improve expression vectors. Here we show that many intermediate promoters also have substantial late promoter activity.

Results

Strategy to selectively express late genes

During VACV infection the transcription of intermediate genes must precede the transcription of late genes because the late

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transcription factors are themselves products of intermediate genes. However, in order to distinguish potential dual intermediate/late promoters from intermediate-only promoters we needed a way to selectively prevent intermediate promoter activity while allowing late promoter activity. Our plan was to (i) construct a cell line that stably expresses late transcription factors, (ii) infect such cells with a VACV mutant that is unable to express intermediate or late transcription factors but will express the viral RNA polymerase, and (iii) transfect the cells with a plasmid containing a luciferase (LUC) reporter gene regulated by the putative intermediate/late or intermediate-only promoter (Fig. 1A). Only plasmids with a late promoter element should express LUC. We already had the VACV intermediate-specific transcription factor deletion mutants vA8Δ and vA23Δ, which are propagated in the cell line RK13-A8/A23 that expresses the two intermediate transcription factors (Warren et al., 2012). However, we still needed a cell line that expresses the three late transcription factors encoded by the G8R, A1L and A2L ORFs (Keck et al., 1990).

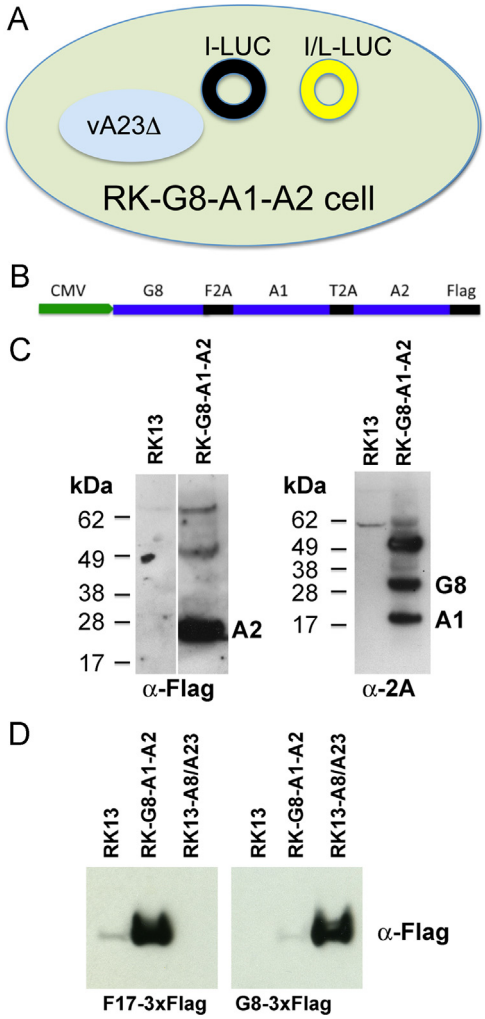

Fig. 1. Construction and characterization of the RK-G8-A1-A2Flag cell line. (A) Diagram depicting the assay to determine dual late promoter activity of intermediate promoters. A cell that expresses the three late transcription factors G8, A1 and A2, is infected with a VACV mutant with a deletion of the A23 intermediate transcription factor and is transfected with a plasmid containing LUC regulated by a strict intermediate (I) promoter or by a dual intermediate/late (I/L) promoter. Black circle indicates no LUC expression; yellow circle indicates LUC expression. (B) Diagram of a tricistronic cassette regulated by a CMV promoter. The picornavirus 2A-like sequences F2A and T2A separate the G8R and A1L ORFs and the A1L and A2L ORFs, respectively. The A2L ORF has a C-terminal Flag tag. (C) Western blots showing G8, A1 and A2 expression. A2 was detected with antibody to the Flag epitope; G8 and A1 were detected with antibody to the 2A peptide. The positions of protein markers in kDa are shown on the left and the A2, G8 and A1 bands on the right. (D) Expression of a late and intermediate gene. RK-13, RK-G8-A1-A2Flag, or RK13-A8/A23 cells were infected with vA23Δ and transfected with plasmids encoding the late F17R and intermediate G8R ORFs with 3XFlag tags regulated by their natural promoters. After 18 h, the cells were lysed and analyzed by Western blotting with anti-Flag antibody. The single flag epitope on the A2 protein was not detected in the small amount of extract used for analysis of the triple flag on the F17 and G8 proteins.

To express G8R, A1L and A2L ORFs in similar amounts in the same cell, we made a tricistronic vector containing the three ORFs separated by picornavirus 2A-like CHYSEL peptide sequences that mediate co-translational separation of the nascent chains (de Felipe and Ryan, 2004; Doronina et al., 2008). A Flag-tag was appended to the C-terminus of the A2L ORF and the tricistronic construct was inserted following the CMV promoter in the pcDNA 3.1/Zeo (+) plasmid (Fig. 1B). RK-13 cells were transfected and Zeocin was used for clonal selection of cells. Western blotting was used to detect expression of the VACV A2Flag protein with antibody to the Flag epitope and the VACV A1 and G8 proteins with antibody to the picornavirus 2A peptide in RK-G8-A1-A2Flag cell lysates (Fig. 1C). High molecular weight bands may represent translational read through.

Confocal microscopy was used to determine the intracellular location of the late transcription factors in RK-G8-A1-A2Flag cells. In uninfected cells, the transcription factors were distributed throughout the cytoplasm (Fig. 2, upper panel). However, when the cells were infected with VACV, the transcription factors apparently migrated into virus factories, which were revealed by staining the DNA with DAPI or with antibody to the A14 membrane protein, which was expressed from the viral genome (Fig. 2, lower panels). The re-localization of all three factors was remarkable and taken as an encouraging sign for their ability to function with the viral RNA polymerase, although the mechanism for sequestering the factors in the factory remains to be determined.

As proof of principal for our strategy, we infected normal RK-13 cells, RK13-A8/A23 cells and RK-G8-A1-A2Flag cells with vA23Δ and transfected them with a plasmid encoding the F17 late protein or the G8 intermediate protein under their natural promoters. Upon Western blotting the F17 protein was detected in the RK-G8-A1-A2Flag cells but not in the RK13-A8/A23 cells, whereas the opposite was true for the G8 protein (Fig. 1D), confirming the function and specificity of the transcription factors and validating the system for our purpose.

Intermediate/late promoter screen

The previously classified 53 intermediate ORFs (Yang et al., 2011b) were used as the starting point for the screen. None of these ORFs were expressed in the presence of AraC indicating that they do not have functional early promoters. However, the presence of late promoter elements remained a possibility. Approximately 80 bp preceding each intermediate ORF was cloned adjacent to the Firefly Luc ORF. Both RK-G8-A1-A2Flag cells and RK-13 cells were infected with vA23Δ and after 1 h were transfected with one of the VACV intermediate promoter Firefly LUC plasmids and with a control plasmid encoding Renilla LUC adjacent to the HSV TK promoter for normalization of transfection efficiency. After 16–18 h, LUC activities were determined in the cell lysates. The non-specific Firefly LUC values in the RK-13 cells were subtracted from the values for the same plasmids in the RK-G8-A1-A2Flag cells. The LUC activities varied in a continuous fashion from very low to values approaching the strong late F17 promoter (Bertholet et al., 1985), which was used as a positive control (Fig. 3, Table 1). We calculated that 26 of the 53 intermediate promoters had more than 5% of the F17 promoter activity.

Correlation of sequence features of intermediate promoters with late promoter activity

Previous studies had pointed out signature features of late promoters: specifically a TAAAT at the RNA start site frequently