



# Kinetic analysis of a complete poxvirus transcriptome reveals an immediate-early class of genes

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**Vaccinia virus is the prototypic orthopoxvirus and was the vaccine used to eradicate smallpox, yet the expression profiles of many of its genes remain unknown. Using a genome tiling array approach, we simultaneously measured the expression levels of all 223 annotated vaccinia virus genes during infection and determined their kinetics. For 95% of these genes, significant transcript levels were detected. Most remarkably, classification of the genes by their expression profiles revealed 35 genes exhibiting immediate-early expression. Although a similar kinetic class has been described for other virus families, to our knowledge, this is the first demonstration of its existence in orthopoxviruses. Despite expression levels higher than for genes in the other three kinetic classes, the functions of more than half of these remain unknown. Additionally, genes within each kinetic class were spatially grouped together in the genome. This genome-wide picture of transcription alters our understanding of how orthopoxviruses regulate gene expression.**

gene transcription | genome tiling array | microarray | vaccinia virus

The orthopoxviruses are a closely related group that share high sequence similarity and many aspects of basic biology (1). This group includes two significant human pathogens, namely, variola virus, the causative agent of smallpox, and monkeypox virus, which is the current cause of emerging disease in central and West Africa. Vaccinia virus (VACV) is the prototypic orthopoxvirus and was the live vaccine used to eradicate smallpox. VACV is now in use as a vector for recombinant vaccines, some of which are in clinical trials.

Like other orthopoxviruses, VACV has a linear, double-stranded DNA genome that is nearly 200 kb long. The most common laboratory strain of VACV, Western Reserve (VACWR), is predicted to encode 223 ORFs. This number includes 12 ORFs (VACWR-001 through VACWR-012 and VACWR-218 through VACWR-207) that are repeated at each end of the genome, leaving a total of 211 unique ORFs.

VACV has a broad cellular tropism *in vitro* and potential host range *in vivo*, but there is no clearly identified animal reservoir for the virus in nature. As a result of an exclusively cytoplasmic life cycle, VACV encode its own enzymes and proteins required for gene transcription, genome replication, virion production, and morphogenesis and, for the most part, does not depend on host cell proteins for these processes. In addition, VACV infection induces a rapid and massive shutdown of host gene expression that acts at several levels (1–4).

Expression kinetics have been described for a variety of VACV genes. These studies, and work done to define promoters and transcription complexes, have led to the definition of four temporal gene classes and three distinct promoter types. The promoters have been named early, intermediate, and late, with each promoter associated with one gene class (5). In addition, some genes have elements of early and late promoters in their

upstream region, giving rise to a fourth class referred to as early/late.

The virion contains early transcription complexes bound to DNA in position to promote transcription of early genes upon entry into host cells (6, 7). Despite analogy with other large, double-stranded DNA viruses, such as herpes viruses, and some use of the term in the literature (8), an immediate-early class of genes has not come to be included in the established model of poxvirus gene expression (5, 9, 10).

In terms of broad functions, besides proteins required for expression of the subsequent gene classes, early genes tend to encode virulence factors for modulation of host responses (11–14). Proteins encoded by late genes are generally required for virion morphogenesis and structure or early gene transcription factors packaged in newly made viral cores (15–17).

Although there is a wealth of information in the literature regarding VACV gene expression, this knowledge is highly fragmented with respect to experimental settings and methodologies used, and substantial gaps exist. Experimental data showing expression kinetics are only available for approximately two-thirds of the annotated ORFs, leaving predictions based on canonical promoter motifs as the only guide for 46 ORFs ([www.poxvirus.org](http://www.poxvirus.org)). For 23 ORFs lacking prototypic promoter sequences, even predictions are not possible.

Although microarrays have been used to show viral gene expression (18, 19), those arrays did not allow resolution between the ORFs and the untranslated regions. Tiling arrays, however, provide a comprehensive and unbiased sampling of transcriptional activity by using overlapping probes covering the entire region of interest (20). In this study, we simultaneously measured transcription of the entire VACV genome during infection and derived a complete picture of gene expression. The close relationship between the orthopoxviruses makes it reasonable to assume that the findings apply across the group (10).

## Results and Discussion

**The Genome Tiling Array Allows for Simultaneous Measurements of VACV Gene Transcription Throughout Infection.** We aimed to determine the temporal transcription profile of all 223 annotated VACV ORFs during infection. HeLa cells were chosen as hosts because they support productive infection and are commonly used for growing VACV stocks (21). Cells were infected at high

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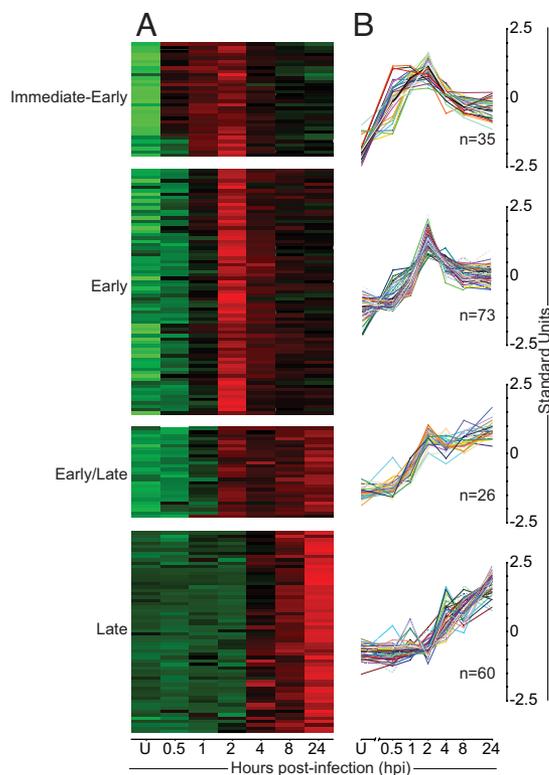
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**Fig. 3.** Cluster analysis of expression profiles revealed an immediate-early class of genes. Genes were divided into four groups based on hierarchical clustering of kinetic expression data. (A) A heat-map representation of ORF expression is shown. Colors indicate the probe intensity level for each gene in uninfected cells (U) and at 0.5–24 hpi; green and red represent lower and higher expression, respectively. (B) Relative expression levels are shown throughout the same time course as in A.

early class was the largest by number, containing 73 genes with the onset of transcription at 1–2 hpi and maximum level of transcription at 2 hpi. A late class of 60 genes was also identified generally expressed from 4 hpi, with levels increasing over time. Finally, an early/late class was found with 26 genes exhibiting onset of transcription typical of early genes but with sustained expression more similar to late genes. Three ORFs (VACWR-033, -063, and -086) did not fit well into any of the classes and were excluded from this part of the analysis.

No “immediate-early/late” gene class was identified, suggesting that immediate-early genes are distinct from the early genes. When early promoter sequences (either verified or putative) for the two classes were compared, only minor differences were seen in the consensus promoter sequences, primarily just upstream of the transcription start site. Thus, although only one type of early promoter has been identified, it is possible that minor changes in promoter sequences or other as-yet-unrecognized sequences allow transcription complexes to preferentially assemble on the promoters of immediate-early genes in virions. Another possibility is that hitherto-unrecognized enhancer elements exist for the immediate-early genes. This is not unreasonable because the alternative would be that the 134 genes with early promoter elements will be transcriptionally active upon initiation of infection, giving the virus relatively poor control over gene expression in the early stages of infection.

We would also like to point out that there is a difference between defining “functional” versus “kinetic” classes of viral genes. Early poxvirus genes are traditionally defined functionally because they are expressed before viral DNA replication. The term immediate-early used here is a kinetic description of

preferential expression before the early genes, and no functional distinction has been made between these classes. Either way, our reidentification of an immediate-early class of genes forces a revision of the current paradigm of poxvirus gene expression.

A second surprise of the cluster analysis was the lack of an intermediate gene class. A few genes in the late class were expressed at maximum levels at 4 hpi, which may suggest that they are intermediate genes. However, even when the analysis was biased in favor of detecting an intermediate class, it was never identified with statistical significance. Genes reported to belong to an intermediate class or having intermediate promoters were either distributed among the immediate-early (VACWR-072), early (VACWR-077), and late (VACWR-119 and VACWR-120) classes, or did not fit well into any class (VACWR-086) (SI Table 2). Some genes with intermediate promoters also have additional promoter elements in their upstream regions (29, 33). The complexity introduced by promoter combinations might explain why they fail to form a distinct cluster. It is also possible that shorter time intervals would be required for distinction between intermediate and late genes (34).

As a test of the biological relevance of the cluster analysis, we examined whether the expression of genes in the late class depended on viral DNA replication. The ORF expression levels were determined at 8 hpi in the presence and absence of Cytosine beta-arabinofuranoside (AraC), a nucleoside analog commonly used to block DNA replication. Of the 60 late genes, the transcription of 52 were either completely ( $n = 33$ ) or partially (reduced by  $\geq 30\%$ ,  $n = 19$ ) blocked upon AraC treatment (SI Table 2). In contrast, transcription was not inhibited by AraC for any of the 134 genes from the other classes (SI Fig. 7). These results confirm that late gene transcription by VACV depends, for the most part, on DNA replication and strengthen the validity of the clustering analysis.

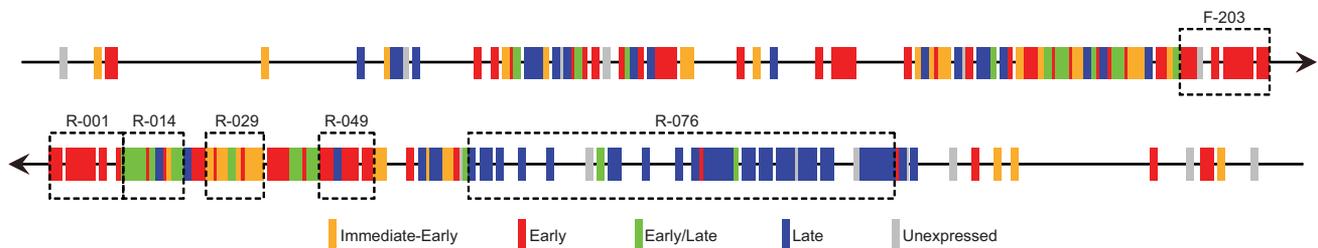
#### Tiling Array Analysis Is Largely Unaffected by Run-On Transcription.

The current data processing does not distinguish between run-on and true transcription. Having assigned genes into kinetic classes, the impact of run-on transcription from upstream genes was assessed at 8 hpi. ORFs were divided into four groups based on the expression kinetics of their upstream neighboring ORF: Early ORFs located after early ORFs, early after late, late after early, and late after late. Median transcript levels for each ORF were compared with those of the 100-nt untranslated regions on either end (SI Fig. 6B). ORFs lacking flanking untranslated regions were not included. This showed that irrespective of the kinetic class of the upstream neighbor, ORF transcripts were, on average, much more abundant than transcripts from the flanking regions. Nevertheless, these analyses will be further refined to perform *de novo* transcript mapping of the virus. That will help to discover novel transcripts and more precisely determine the boundaries of transcription initiation and termination.

#### Functions of Genes in the Different Kinetic Classes.

After the clustering of the genes by their expression profiles, a detailed analysis of gene functions within each kinetic class was performed. Each gene was assigned to one of five functional categories based on literature annotations and predicted functions (SI Table 3): DNA replication, immune evasion/virulence, transcription, virion core proteins, and virion membrane proteins (SI Table 2). Genes of unknown function and genes with functions outside of these categories were excluded. In addition, for ORFs that are fragments of a larger ancestor gene, only the ORF proximal to the promoter was placed into the corresponding category, and the downstream ORF(s) were denoted as “pseudo” and excluded. The analysis showed marked differences between genes in the two earliest classes, which were mainly involved in DNA replication, evasion/virulence, and transcription, and the early/late and late genes, which had a larger proportion of genes





**Fig. 5.** Genes of different kinetic classes are clustered in the genome. Temporal expression is shown for each gene along the two strands of the VACV genome. The genes are positioned in their proper order, but sizes and spacing have been drawn identical for all ORFs. Boxes denote regions with an overrepresentation of genes from a certain kinetic class. F/R indicates the strand and numbers represent the VACWR name of the first ORF in each region.

To get an overview of transcription throughout the genome, a map of the transcriptome was constructed (Fig. 5). Visual inspection supported by statistical analysis (see *Materials and Methods*) revealed a strong preference for colocalization of genes within the same kinetic class. For all four classes, the occurrence of a neighboring gene within the same kinetic class was significantly higher than expected (SI Table 4). Six regions were identified in which genes of a certain class were significantly over-represented with respect to their overall genomic distribution (Fig. 5).

A further extension of this broad gene expression approach would be to analyze the expression at the protein level and combine these data with the rapidly increasing amount of information about immunogenicity of VACV proteins [www.immuneepitope.org (41)]. This may allow us to unravel the rules that underpin immunogenicity and immunodominance in the host response to large pathogens. Last, a comprehensive study encompassing viral and host genes, especially those of immunological relevance, will help reveal the interactions between these two players in pathogenesis.

We have rendered the first complete picture of an orthopoxvirus transcriptome and demonstrated that the vast majority of annotated VACV genes are expressed. This also led to the surprising discovery of an immediate-early class of genes, more than half of which have unknown function despite being expressed at very high levels. Our study demonstrates the power of a genome-wide approach, compels a revision of the current understanding of orthopoxvirus gene regulation, and suggests many lines of investigation in orthopox virology and pathogenesis.

## Materials and Methods

**Viral Stocks.** The WR strain of vaccinia virus was obtained from B. Moss (National Institutes of Allergy and Infectious Diseases, Bethesda, MD).

**Infection and Flow Cytometry.** HeLa cells were incubated at  $10^7$  cells per  $100 \mu\text{l}$  complete media with VACWR at an MOI of 10:1. After 60 min,  $30 \times 10^6$  cells were distributed to  $225\text{-cm}^2$  flasks with 30 ml of complete media and cultured at  $37^\circ\text{C}$ . The method of infection was tested by using a GFP-encoding VACV42 and/or staining with an anti-VACV serum (ViroStat) and analyzed by flow cytometry. AraC was used at  $40 \mu\text{g/ml}$  during infection in some samples to block viral DNA replication. Expression was considered significantly decreased when median signal intensity was  $\geq 30\%$  reduced. Productive infection or replication block were tested by enumerating the plaques formed in the AraC-treated samples at 2 and 24 hpi.

**RNA Preparation, Labeling, and Hybridization.** Cells were harvested and resuspended in TRIzol and purified according to manufacturer's protocol. RNA clean-up was performed by using RNeasy columns. Ribosomal RNA was depleted by using the RiboMinus transcriptome isolation kit. NA samples were chemically labeled with biotin, using the ULS aRNA labeling kit, and hybridized to the arrays. Arrays were scanned by using the Affymetrix GeneChip Scanner 3000 7G and standard Affymetrix protocol as described in ref. 43.

**Q-PCR.** Q-PCR was performed as described in ref. 44. For each primer set, expression levels were quantified relative to that of human 18S rRNA. Con-

firmation of single amplicons and lack of primer-dimers were performed by melting-curve analysis and gel electrophoresis. Samples prepared in the absence of reverse transcription were run for each primer pair to confirm specificity and to exclude that the signal came from contaminating genomic DNA.

**Affymetrix GeneChip NimbleExpress Tiling Array Design.** The genome tiling array was built with NimbleGen probe synthesis technology. Packaging of the array was developed in collaboration with Affymetrix, using 25-mer probes covering both strands of the VACV genome (NCBI: AY243312.1) with a 4-nt spacing (97,334 probes). The array includes 15,308 negative control probes specific for *Arabidopsis thaliana* and 14,399 synthetic "antigenomic" probes with varying GC content.

**Background Subtraction and Data Normalization.** Probe level data were log<sub>2</sub>-transformed to stabilize their variance. The fluorescence signal resulting from nonspecific hybridization was subtracted by using the synthetic and *A. thaliana* probes as empirical estimators. As a strong dependence of nonspecific signal on GC content was observed (SI Fig. 9), the median background probe signal at the corresponding GC content was subtracted from the signal of each VACV probe. Probes with GC content  $< 3$  nt were not well represented and were grouped. After removal of nonspecific background, a dependence of the specific signal on GC content was still apparent. This was minimized by mapping all probe signals to their corresponding values in the empirical distribution of probes with a GC content equal to 8. To enable the direct comparison of signal intensities across different arrays, probe signals were quantile-normalized (45). For replicate samples, quantile-normalized signals for each probe were averaged.

**Data Summarization and Significance Testing.** Median quantile-normalized probe intensities for probes lying completely within each ORF were used as representative ORF signal intensity. Probes for identical ORFs (VACWR-001 through VACWR-012 and VACWR-218 through VACWR-207) were combined. The significance of signal intensity in each ORF was calculated by using the binomial distribution (20).  $P \leq 10^{-5}$  was used as a threshold, because this approximately corresponds to a value of  $10^{-3}$  after adjusting for the number of tests performed ( $n = 223$ ).

**Conversion to Standard Units.** To observe the correlations between temporal expression patterns, the data were converted to standard units: Standard Units =  $(S - \mu)/\sigma$ .  $S$  = probe signal,  $\mu$  = mean probe signal over the time course, and  $\sigma$  = standard deviation.

**Gene Clustering and Bootstrapping Analysis.** Standardized expression profiles for each ORF were clustered by using the "cutHclust" function of the Class-Discovery library (46) in the R statistical programming language (47). Data from 1–24 hpi were used. Data from uninfected cells and 0.5 hpi were excluded as their signal intensities were considerably lower and introduced excessive noise. All parameters were left at default values except for  $k$ , which was varied from 2 to 7. The "BootstrapClusterTest" function was used with values of  $k$  in the same range to determine the number of biologically relevant clusters. Other parameters included: cutHclust, metric = Pearson, and  $n$ Times = 100. Data from 10 iterations were pooled. The robustness of clusters was measured averaging the number of times that two patterns ended up in the same cluster through 1,000 iterations on a subset of the data. A Pearson correlation test was run comparing expression values for each gene with the average for each cluster.  $R > 0.7$  was considered as significant.

**Gene Colocalization Statistics.** The cluster of each gene and the kinetics of its neighbors was noted in genomic space. The position of each gene was shuffled randomly 10,000 times while keeping track of the neighboring genes' kinetics during each iteration. Each pairwise distribution of neighboring genes was calculated in this manner. The observed frequency of colocalization was converted into a Z score (and P value), using standard deviations obtained through the randomization process.

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