INTRODUCTION

Vaccinia virus is a member of the poxvirus family which is characterized by large DNA genomes and the ability to compete replication in the cytoplasm of eukaryotic cells. The poxvirus replication cycle is divided into three stages of gene expression that are temporally regulated at the level of transcription initiation, resulting in the sequential expression of early, intermediate and late genes (reviewed by Broyles, 2003). Poxviruses encode many proteins required for nucleic acid biosynthesis, including a multi-subunit RNA polymerase that is believed to transcribe all viral mRNAs. Viral proteins in purified forms are sufficient to transcribe early genes and synthesize viral DNA. Viral proteins also participate in the transcription of intermediate and late promoters. Three viral factors, the viral capping enzyme, the E4L gene product and VITF-3, are necessary for the transcription of intermediate genes (Rosales et al., 1994a, b; Sanz & Moss, 1999). Late transcription also requires three viral factors, the A1L, A2L and G8R gene products (Keck et al., 1990), and has been shown to be stimulated by the H5R gene product (Kovacs & Moss, 1996). Recapitulation of intermediate and late transcription in vitro has implicated several cellular proteins that stimulate transcription of intermediate and late genes. VITF-2 stimulates intermediate transcription and has been identified as the heterogeneous ribonucleoprotein SH3-domain-binding protein (G3BP) and the cytoplasmic activation and proliferation-associated protein (p137) (Katsafanas & Moss, 2004). Two cellular proteins identified as the heterogeneous ribonucleoproteins A2/B1 and RBM3 have also been reported to activate transcription of late genes in vitro (Wright et al., 2001). The contributions of these cellular proteins to vaccinia transcription remain unclear. Recent studies have suggested that YY1 is an activator of vaccinia virus transcription mediated by the INR in viral promoters (Broyles et al., 1999). YY1 is a multifunctional transcriptional regulator ubiquitously expressed in metazoans and can function as an initiator, activator or repressor of transcription depending on its context in different promoters (reviewed by Galvin & Shi, 1997; Gordon et al., 2006). The 414 aa protein has an N-terminal activation domain and C-terminal DNA-binding domain composed of four zinc finger structures that also interacts with several basal transcription factors, coactivators and corepressors to differentially regulate the transcription process. Significantly, YY1 has been demonstrated to localize with vaccinia virus replication complexes in the cytoplasm of cells in a manner that requires its DNA-binding domain (Oh & Broyles, 2005).

Downregulation of vaccinia virus intermediate and late promoters by host transcription factor YY1

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Approximately half of the intermediate and late gene transcriptional promoters of vaccinia virus have a binding site for the cellular transcription factor YY1 that overlaps the initiator elements. Depletion of YY1 using RNA interference enhanced the activity of these promoters, while overexpression of YY1 repressed their activity. Viral promoter nucleotide replacements that specifically impair the binding of YY1 mostly alleviated the transcriptional repression and correlated with the ability of YY1 to stably interact with the initiator DNAs in vitro. The transcriptional repression activity was localized to the C-terminal DNA-binding domain of the protein. These results indicate that YY1 functions to negatively regulate these vaccinia virus promoters by binding to their initiator elements.
In this study, the role of YY1 in vaccinia virus post-replicative transcription has been pursued in more detail. Several lines of evidence are presented to support a role for repression of vaccinia virus transcription by YY1 rather than activation, as previously suggested. The YY1-mediated repression of intermediate and late promoters may serve as a mechanism to delay their expression.

**METHODS**

**Cell culture, virus infection and DNA transfections.** HeLa S-3 cells were grown in monolayers in 6-well (9 cm²) or 12-well (3.7 cm²) trays in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine serum. Cells were infected with 10 p.f.u. per cell vaccinia virus Western Reserve strain. After 1 h virus infection, plasmid transfections were performed in triplicate with the indicated amounts of DNA per well, using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Virus yields were determined by adsorption of virus (10 p.f.u. per cell) on HeLa cells for 1 h followed by washing cell monolayers with medium twice. Cells were harvested at the indicated times and lysed by two cycles of freeze–thawing. Virus titres were determined using BSC40 cells (Broyles et al., 2004).

**YY1 RNA interference.** A synthetic 21 nt YY1 short interfering RNA (siRNA) duplex (Dharmacon) was designed against the target YY1 mRNA sequences 5’-GCAGCAAACAUUGAGAAA-3’ and 5’-UUGUCUAGAUGCGd-3’. Green fluorescent protein (GFP) siRNA (Leirdal & Sioud, 2002) was also chemically synthesized as a non-specific control.

siRNA transfections were performed with 20 μM duplex RNA and 10 μl oligofectamine transfection reagent (Invitrogen) according to the manufacturer’s instructions. HeLa cells were seeded in either 6- or 12-well plates at 40–50% confluence on the day of transfection.

After 3 days, cells were infected with virus and transfected with reporters as described above.

**Plasmids.** Histidine-tagged YY1 was a gift from Thomas Shenk (Princeton University) and the YY1 truncation mutants were gifts from Bernhard Lüscher (Universitätsklinikum der RWTH). Site-directed mutagenesis was performed as described by Tyagi et al. (2004).

Wild-type YY1, truncation and point mutants were expressed from the vector pBl-11HA; this is driven by the vaccinia I1L promoter, a strong intermediate-class promoter (Liu et al., 2004). Reporter plasmids were constructed by ligating double-stranded synthetic oligonucleotides (Integrated DNA Technologies) with the desired sequence upstream of the Escherichia coli β-galactosidase gene. Plasmids were purified using Qiagen columns.

**Reporter assay.** Cells infected with vaccinia virus and transfected with the desired plasmids were harvested 16–18 h (unless otherwise stated) after transfection, washed with PBS (150 mM NaCl, 15 mM Na₂HPO₄, pH 7.4) and lysed on ice with intermitting vortexing for 20 min in 0.3–0.5 ml lysis buffer (0.1 M Tris/HC1, pH 8.0, 0.5% Triton X-100). Cellular debris was removed by centrifugation, and the supernatant was used for subsequent steps. Reporter enzyme assays for β-galactosidase were performed in duplicate or triplicate with o-nitrophenyl-β-D-galactoside as the substrate as described previously (Miller, 1972). Enzyme activity was normalized to protein content which was determined with Bradford’s reagent (Bio-Rad).

**Recombinant YY1 protein purification.** Native histidine-tagged YY1 was expressed in E. coli DE3 cells. The protein was purified by adsorption onto Ni²⁺-nitriloagarose resin (Qiagen) that was eluted between 200 and 300 mM imidazole.

**Electrophoretic gel mobility shift assays.** DNA oligonucleotides were synthesized chemically (Integrated DNA Technologies), labelled at their 5’ ends with T4 polynucleotide kinase and [γ-³²P]ATP [3000 Ci (110 000 TBq) mmol⁻¹], and annealed to form duplex DNA with blunt ends. The following INR DNA sequences were used: I1LWT, 5’-GCAAGTTGATTTAAAAAGTTGTTGGA-3’; I1L+6GAA, 5’-GCAAGTTGAACTTAAAAATGGCCGGA-3’; G8RWT, 5’-GCAAGAAAATTTTTAATGAGCAGGA-3’; G8R+6AAG, 5’-GCAAGAAAATTTTTAATGAGCAGGA-3’; A26LWT, 5’-GCAAGTTGATTTAAAAATGGCCGGA-3’; A26L+6GAA, 5’-GCAAGTTGATTTAAAAATGGCCGGA-3’; F17RWT, 5’-GCAAGTTGATTTAAAAATGGCCGGA-3’; F17R+6AAG, 5’-GCAAGTTGATTTAAAAATGGCCGGA-3’.

**Immunoblotting.** Total cell proteins were separated by electrophoresis on a 12% SDS polyacrylamide gel and transferred onto PVDF membrane.

Blots were probed with antibody directed against the C terminus of YY1 (sc-281; Santa Cruz), and with β-actin (A5316; Sigma), and visualized with enhanced chemiluminescence reagents (Amersham).

**RESULTS**

**The effect of depletion of YY1 with siRNAs on vaccinia virus intermediate and late transcription**

Four vaccinia virus promoters were chosen for this study: the intermediate I1L and late A26L promoters, which have a consensus YY1 binding site (CCATTG) overlapping their INR with the dinucleotide GG at positions +5 and +6, and the intermediate G8R and late F17R promoters, which have an A residue at position +6 and therefore do not conform to the consensus YY1 binding site (Fig. 1). Each of these promoters was inserted upstream of a β-galactosidase reporter gene to evaluate promoter activity.

In order to establish the functional role of YY1 in the regulation of vaccinia virus intermediate and late gene expression, RNA interference was used to deplete cellular pools of YY1. siRNAs were designed against the YY1 mRNA sequence and transfected into HeLa cells. YY1-specific siRNAs reduced YY1 protein levels by 70–80% relative to control GFP siRNAs, using β-actin as a loading control (Fig. 2a). After YY1 depletion was achieved, cells were infected with either a recombinant vaccinia virus bearing the β-galactosidase gene driven by the I1L promoter or wild-type virus and then transfected with a plasmid bearing the same I1L promoter-β-galactosidase cassette. Depletion of YY1 protein levels by siRNA transfection resulted in approximately double the promoter activity.
in both cases (Fig. 2b). It was concluded that transfected plasmids with viral promoters respond to YY1 depletion similarly to a promoter in the viral genome.

Representative vaccinia promoters were evaluated for their response to YY1 depletion using transfected plasmids as a function of time after virus infection. The activity of the I1L promoter was elevated by about 70% at 4 h post-infection (p.i.), relative to that of the GFP-control-siRNA-transfected cells (Fig. 2c). In HeLa cells depleted of YY1, the activity of the late A26L promoter was elevated by about 60% at 6 h p.i., and remained elevated throughout the course of the 18 h experiment (Fig. 2d). The activity of the intermediate G8R and late F17R promoters, each having an A residue immediately following the initiator element and therefore lacking a canonical YY1 binding site, was relatively unaffected by the YY1 siRNAs (Fig. 2c, d). These results indicate that depletion of YY1 from HeLa cells leads to enhancement of vaccinia intermediate and late promoters containing a YY1 binding site at their INRs.

The effect of overexpression of YY1 on activity of vaccinia virus intermediate and late promoters

To induce increased levels of YY1 in the cell, the YY1 gene was inserted downstream of a vaccinia promoter and cotransfected with reporter genes into virus-infected cells. Increasing amounts of YY1 expression vector in transfections reduced the activity of the I1L promoter in a dose-responsive manner over the course of 24 h (Fig. 3a). The activity of the intermediate I1L promoter was reduced by 90% by expression of high levels (e.g. transfection with 0.75 μg plasmid) of YY1 (Fig. 3b). Overexpression of YY1 also inhibited the activity of the A26L promoter to a similar extent (Fig. 3b). YY expression had less effect on the activities of the intermediate G8R and late F17R promoters whose activities were reduced by about 30 and 10%, respectively. These findings indicate that YY1 can act as a transcriptional repressor of vaccinia virus transcription provided that the promoter’s INR has an overlapping YY1 binding site.

A functional YY1 binding site in a promoter INR element is required for repression of intermediate and late promoters

A possible interaction of endogenous YY1 with vaccinia virus promoters was investigated by altering the +6 nucleotide of the promoters. When vaccinia-infected cells were transfected with reporter gene constructs alone, the activity of the I1L promoter was enhanced by 20%, and the A26L promoter by 25% when the G residue at the +6 position of the promoter was replaced with A (Fig. 4). The
activity of both of the altered promoters was highest at times expected for the peak of transcription. At later times, the activity of both decreased. These results suggest that endogenous levels of YY1 are capable of repression of activity of promoters that have a YY1 binding site at their INR elements. For reasons that are unclear, the converse experiment, in which a G residue was introduced at nucleotide +6 of the G8R and F17R promoters, did not affect their activities (data not shown).

As a further test of the importance of the +6 G nucleotide, the response of the vaccinia promoters to overexpression of YY1 was monitored. As a control, a YY1 mutant with K339S and R342S amino acid replacements in the second zinc finger which specifically impair DNA binding (Austen et al., 1998) was also expressed. This mutant is particularly useful for discriminating effects due to DNA binding because its known interaction with other proteins is apparently normal. Replacement of G at nucleotide +6 of the intermediate I1L promoter with an A residue, designated +6GΔA, did not significantly affect its activity under the conditions tested here (Fig. 5a). Expression of

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**Fig. 3.** Effect of overexpression of YY1 on the activity of vaccinia intermediate and late promoters. (a) HeLa cells were infected with vaccinia virus and transfected with empty vector (●) or 0.25 (■), 0.5 (■) or 0.75 (□) µg YY1 expression vector and co-transfected with plasmid with the β-galactosidase gene driven by the viral I1L promoter. Cells were harvested at the indicated times p.i., and reporter enzyme activity was determined. (b) HeLa cells were infected with vaccinia virus and co-transfected with the indicated promoter–reporter plasmid and YY1 expression vector (empty bars) or empty vector (filled bars). Cells were harvested 16 h after infection. Error bars, SD.

**Fig. 4.** Effect of substituting nucleotide +6 in the YY1 binding site of vaccinia virus promoters. The G nucleotide at position +6 of the I1L promoter (a) or A26L promoter (b) was replaced with an A. HeLa cells were infected with vaccinia virus and transfected with vectors driven by the indicated promoter. At the indicated times, cells were harvested and reporter enzyme levels were determined. Relative activities are expressed as the activity of the +6GΔA promoter relative to that of the promoter of the native sequence. Error bars, SD.

**Fig. 5.** Effect of the +6GΔA and +6AΔG substitutions on the response of the I1L, G8R, A26L and F17R promoters to overexpression of YY1. HeLa cells were infected with vaccinia virus and co-transfected with reporter gene plasmids driven by the I1L (a), A26L (b), G8R (c) or F17R (d) promoters with native sequence (filled bars) or with +6GΔA (a, b) or +6AΔG (c, d) replacement (empty bars) as well as native YY1 expression vector (WT), YY1 KR mutant expression vector (KR) or empty vector (EV). Cells were harvested at 16 h p.i. and reporter enzyme levels were determined. Expressed promoter activities are those of the +6GΔA or +6AΔG replacements relative to that of the native promoter at the same time point (%). Errors bars, SD.
YY1 severely inhibited the native I1L promoter, consistent with results described above. Expression of wild-type YY1 reduced the activity of the I1L +6GΔA promoter by about 20%, similar to that observed by expression of the YY1 K395S, R342S mutant. Thus, the +6GΔA nucleotide replacement significantly reduced the response of the I1L promoter to YY1 expression. A similar response was observed for the late A26L promoter which also has a YY1 binding site overlapping its INR (Fig. 5b). The repression of A26L promoter activity by YY1 expression was largely alleviated by the +6GΔA nucleotide replacement. The converse experiments were performed with the intermediate G8R and late F17R promoters which lack an apparent YY1 binding site. After an A→G substitution at the +6 position, the promoters were repressed partially by YY1 expression, whereas the native sequence promoters were relatively insensitive to YY1 (Fig. 5c, d). It is noted, however, that the repression of promoter activity from the converted G8R and F17R promoters was not as severe as with promoters that naturally have a G at position +6. Nonetheless, it is concluded that a core YY1 binding site must be present for a vaccinia promoter to be repressed by YY1.

To verify the interaction of YY1 with the various promoter INR elements discussed above, electrophoretic mobility shift assays were performed with radiolabelled oligonucleotides bearing the sequence of the vaccinia INRs and recombinant YY1. Titration of the intermediate I1L and late A26L INR oligonucleotides with YY1 produced a protein–DNA complex of distinct mobility (Fig. 6a). These complexes were not observed when the +6 G residue was replaced with A. The G8R promoter oligonucleotide produced a weak complex with YY1, and the late F17R promoter oligonucleotide yielded no detectable complex (Fig. 6b). Protein complexes with both the G8R and F17R promoters were significantly enhanced when the A residue at the +6 position of the promoters was replaced with a G. Based on these results, it was concluded that the G residue at the +6 position of intermediate and late promoters is essential for stable interaction with YY1.

**The C-terminal DNA-binding domain of YY1 is responsible for repression of vaccinia virus intermediate and late promoters**

YY1 is a modular transcription factor with a four-zinc-finger DNA-binding domain at its C terminus (Shi et al., 1991). Segments of YY1 were expressed here as the C-terminal domain (CTD) containing the four zinc fingers and as the N-terminal domain (NTD) which constituted the remainder of the protein excluding the zinc fingers, for comparison with the activity of the full-length protein. As described above, expression of full-length YY1 reduced the activity of the intermediate I1L promoter and late A26L promoter (Fig. 7a, b). Expression of the N-terminal domain alone had little effect on reporter gene activity for either promoter. A titration experiment demonstrated that the expression of the CTD repressed reporter gene activity to approximately the same extent as the full-length protein (Fig. 7c). It was concluded that the CTD of YY1 is responsible for the inhibitory effects of the protein on vaccinia virus promoters.

![Fig. 6. Effect of the +6GΔA and +6AΔG substitutions on binding of YY1 to intermediate and late INR elements. Recombinant YY1 was incubated with oligonucleotide containing the INR of the I1L and A26L promoters (a) and G8R and F17R promoters (b). Binding mixtures were separated by electrophoresis on a polyacrylamide gel to resolve protein–DNA complexes, indicated by arrows. Lanes: 1 and 8, oligonucleotides alone; 2, 5, 9 and 12, oligonucleotides incubated with 50 ng YY1; 3, 6, 10 and 13, oligonucleotide incubated with 100 ng YY1; and 4, 7, 11 and 14, oligonucleotides incubated with 200 ng YY1. WT, INR elements of native sequence; +6GΔA and +6AΔG, promoters with the nucleotide replacement at the +6 position of the INR element. Faster migrating protein–DNA complexes that contain an N-terminally truncated form of YY1 are indicated by an asterisk.](image-url)
Effect of YY1 depletion on virus growth

If YY1 has a repressive effect on the transcription of large numbers of vaccinia virus genes, it may also have a generally repressive effect on virus growth. To test this possibility, YY1 depletion was used again to assess its effect on virus yield from cells infected with a range of p.f.u. per cell. YY1-depleted cells, along with control siRNA (GFP)-treated cells were infected with vaccinia virus for 24 and 48 h, and virus yields were determined. The amount of virus recovered from YY1-depleted cells was 25 and 15% increased relative to GFP-siRNA-treated cells at 24 and 48 h p.i., respectively, at 10 p.f.u. per cell (Table 1). Moderately greater effects of YY1 depletion were observed at 0.1 and 1 p.f.u. per cell. This result suggests that YY1 has a negative influence on viral replication.

DISCUSSION

The results presented here demonstrate that YY1 depletion from cells resulted in enhanced promoter activity from vaccinia virus intermediate and late promoters, and that overexpression of YY1 repressed the same activities, provided that a G residue is present at position +6 of the promoter to constitute a functional YY1 binding site in the INR element. The isolated DNA-binding domain of YY1 was capable of recapitulating the repressive effects of the full-length protein. In a previous study, the DNA-binding domain of YY1 was shown to co-localize with vaccinia replication complexes in the cytoplasm of infected cells, just as the full-length protein does (Oh & Broyles, 2005). Taken together, these findings indicate that YY1 represses vaccinia transcription through binding to post-replicative promoter INR elements. Vaccinia post-replicative promoters have a relatively rigid sequence requirement in the INR element for the nucleotides TAAAT at positions −1 to +4 on the non-template strand of the helix (Baldick et al., 1992; Davison & Moss, 1989; Knutson et al., 2006).

While the basis for this sequence requirement is not fully understood, the viral RNA polymerase must interact with these nucleotides because initiation of transcription occurs within the three A residues to generate a non-template-encoded oligoadenylate sequence at the 5′ end of the mRNA by an apparent slippage mechanism (Bertholet et al., 1987; Schwer et al., 1987). TBP has been reported to bind INR elements as well as core elements (Knutson et al., 2006). Promoter mutational analysis, however, showed that promoter activity and the interaction of TBP with the INR element could be uncoupled, casting doubt on the importance of the interaction of TBP with INR elements. Therefore, it seems reasonable to propose that YY1 exerts its repressive effects on vaccinia transcription by precluding interaction of the RNA polymerase with the INR elements that overlap the YY1 binding site. A YY1 molecule occupying an INR element could sterically block an incoming RNA polymerase. Alternatively, YY1 could engage an RNA polymerase preinitiation complex to prevent the polymerase from exiting the promoter in the transcription initiation process. Because YY1 contacts purine and pyrimidine residues in the major groove of its core recognition sequence (Houbaviy et al., 1996), steric hindrance of RNA polymerase by YY1 seems more likely.

The kinetics of reporter gene activity in vaccinia-virus-infected cells seem to provide much information about the plausible role for YY1 in vaccinia transcription. In the absence of YY1 overexpression, the +6GAA nucleotide replacement resulted in upregulation of the I1L and A26L...
promoters (Fig. 4). The stimulatory effects were most prominent early in the infectious cycle, and waned thereafter. The nucleotide replacement that would render the promoters largely resistant to the effects of YY1 enhanced their activity in the earlier hours of infection, but normal activity later resumed. These results suggest that subclasses of promoters might exist, effectively defining a delayed intermediate and delayed late class of vaccinia virus promoters as a result of YY1 intervention. Approximately half of the vaccinia virus promoters contain a YY1 binding site overlapping their initiator elements, suggesting that YY1-mediated repression has a global effect on viral gene expression and propagation. Delaying expression of a subset of genes may be necessary to create an additional level of regulation that allows the appropriate timing and amount of viral gene products over the late stages of poxvirus infection. Activities of the G8R and F17R promoters were not enhanced by the G substitution at the +6 position when transfected alone into vaccinia-virus-infected cells. YY1 initiator DNA-binding assays revealed that YY1 has much weaker affinity for these promoters were not enhanced by the G substitution at the +6 position when transfected alone into vaccinia-virus-infected cells. YY1 initiator DNA-binding assays revealed that YY1 has much weaker affinity for these promoter which has been shown to be repressed by YY1 through interaction with the INR unless adenovirus E1A protein is present (Seto et al., 1991). In general, YY1 is believed to mediate its effects through cooperation with other proteins functioning in transcription. Among these proteins is TBP, which can cooperate with YY1 to activate or repress transcription (Gordon et al., 2006). We have reported previously that TBP is the protein responsible for targeting the upstream core element in vaccinia intermediate and late promoters (Knutson et al., 2006). It is possible that YY1 assembles onto INR elements through cooperation with TBP that is bound upstream. The details of the interaction of these two proteins would probably be different in intermediate and late promoters because the spacing between the core and INR elements in these promoters differs by about one turn of the DNA helix (Knutson et al., 2006). In this regard, YY1 has been reported to bend DNA upon interaction with its cognate site (Kim & Shapiro, 1996; Natesan & Gilman, 1993). DNA bending could have significant contributions to the assembly of protein–protein complexes on DNA. TBP also introduces a sharp kink when bound to DNA (Burley & Roeder, 1996). If YY1 and TBP interact on DNA simultaneously, the resulting assembly could be complex and difficult to predict. Clearly, additional studies are required to characterize the mechanism of the repression of vaccinia transcription by YY1.

The increased virus yield when cells were depleted of YY1 indicates that YY1 has a negative influence on virus replication. The increase was mild, however, further indicating that the virus and YY1 can coexist to allow a productive infectious cycle.

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REFERENCES


