Transcription Factor YY1 Is a Vaccinia Virus Late Promoter Activator*

Steven S. Broyles‡, Xu Liu, Min Zhu, and Marcia Kremer

From the Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907-1153

Vaccinia virus has a DNA genome, yet replicates in the cytoplasmic compartment of the cell. We previously described the identification of a cellular protein having high affinity for vaccinia virus late promoter DNA. Sequence substitutions in the vaccinia IIL promoter were used to define a 5-nucleotide block at the transcription initiation site as essential for interaction with the protein. Within this sequence is the recognition motif for the nuclear transcription factor YY1. This factor regulates a multitude of cellular promoters, as an activator of transcription, as a repressor, or as an initiator element-binding protein. Antibodies directed against YY1 were used to show that YY1 copurified with the vaccinia late promoter-binding protein and was present in late promoter-protein complexes in gel supershift assays. Bacterially expressed YY1 also bound specifically to late promoter DNA. A dinucleotide replacement within the YY1 recognition motif directly adjacent to the transcription start site severely reduced the affinity of YY1 for the IIL promoter in vitro and impaired IIL promoter-dependent transcription in vivo. The intracellular localization of YY1 was shown by immunofluorescence microscopy to shift from primarily nuclear to the cytoplasm after vaccinia infection. These results indicate that YY1 has a positive role in the regulation of vaccinia virus late gene transcription and suggest that poxviruses have adapted cellular initiator elements as a means of regulating viral gene expression. This is the first identifiable cellular protein implicated in poxvirus transcription.

Poxviruses are large DNA genome viruses that replicate in the cytoplasm of host cells. Many species of insects, birds, and mammals including humans are natural hosts for specific members of this large family of viruses. As the laboratory prototype for poxviruses, vaccinia virus is by far the best characterized of the group. Vaccinia has a 191-kilobase pair genome encoding some 200 different proteins (reviewed in Ref. 1). Part of the complexity of the virus owes to its battery of genes encoding anti-inflammatory and immunity-suppressing proteins important for infectious spread in an animal host. Many other proteins are made essential by the fact that the virus replicates outside the nucleus in the cytoplasm of the cell. Vaccinia is able to synthesize its mRNA and DNA outside the nucleus, suggesting that the virus must either encode its own proteins participating in nucleic acid metabolism or alternatively recruit nuclear proteins to replicate virosomes in the cytoplasm. Because of the paucity of information on host proteins participating in viral mRNA and DNA synthesis, it is generally believed that vaccinia encodes the majority of proteins functioning in these processes.

The life cycle of vaccinia virus is controlled at the level of the timing of transcription of individual genes (2). Viral genes fall into three classes: early, intermediate, and late. About half of the virus' genes are early and half are late. Only five genes are known to be intermediate. Each class has its own requisite set of transcription factors that regulate the viral RNA polymerase that structurally and functionally resembles its cellular counterparts. The products of the intermediate genes A1L, A2L, and G3R are required for the subsequent activation of the late class of genes (3), and the H5R gene product stimulates late transcription in vitro (4). A cellular protein has also been described as being essential for transcription in vitro (5, 6). It is not yet known what proteins target late promoters for initiation of transcription.

In a previous report, we described a cellular protein with vaccinia late promoter binding activity (7). Prior indications were that the protein interacts with DNA around the start site for transcription in a viral late promoter and was capable of stimulating transcription in vitro. Here we report that the late promoter-binding protein is the transcription factor YY1. Evidence is provided for a positive role for YY1 in transcription from a vaccinia late promoter in vivo, and it is shown that vaccinia infection redirects the subcellular localization of YY1 from the nucleus to the cytoplasm.

EXPERIMENTAL PROCEDURES

Protein Purification—The protein was purified from HeLa cells grown in suspension culture. Cell fractionation experiments indicated that greater than 90% of the late promoter-binding protein could be recovered in nuclear extracts relative to cytoplasmic fractions. Nuclei were isolated and extracted with 0.6 M KCl, 50 mM Tris, pH 8, 0.01% Nonidet P-40 (8). The extract was applied directly onto nickel-agarose as a binding plasmid pHIS-YY1 (provided by Thomas Shenk, Princeton University) as described previously (9). Cells were lysed by freeze-thawing, and YY1 was purified from the soluble fraction on nickel-agarose as described above.

Protein Assays—DNA binding was assayed by electrophoretic gel shift using the vaccinia virus I1L or H1L promoter DNA excised from a...
plasmid or annealed synthetic oligonucleotides and end-labeled with $^{32}$P (10). Protein (5–10 ng for experiments utilizing purified protein) and 1–2 ng of radiolabeled DNA probes were incubated together in the presence of 0.1 μg of poly(dI-dC), electrophoresed on a low ionic strength 4% polyacrylamide gel, and visualized by autoradiography (11). Gel supershift assays, 2 μg of anti-YY1 antibody (Santa Cruz Biotechnology) was included in the DNA binding reaction prior to loading onto the gel. The antibody used in these studies is a rabbit IgG directed against the C-terminal 20 amino acids of YY1.

Western blotting of proteins was as described (12) after electrophoresis on a 10% polyacrylamide gel containing 0.1% SDS. Blots were probed with 5 μg/ml anti-YY1 antibody.

**Reporter Gene Experiments**—The I1L promoter (nucleotides −29 to +16, relative to the 5′-A in the TAAAT motif) was ligated in the form of an annealed double-stranded oligonucleotide into the KpnI and NcoI sites of plasmid pTM1 to place the β-galactosidase gene behind the I1L promoter. Mutation of the promoter was achieved by a 3-fragment polymerase chain reaction strategy (13). For reporter gene expression, 1 × 10⁶ HeLa cells were grown to 60% confluent monolayers and infected with vaccinia virus WR strain at a multiplicity of 10 pfu/cell. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; 150 mM NaCl, 15 mM sodium phosphate, pH 7.0) for 10 min at 22 °C and were permeabilized by washing three times with 0.2% Triton X-100 in PBS for 5 min (12). Nonspecific binding sites were blocked by incubation with 2 mg/ml fluorescein isothiocyanate-conjugated anti-rabbit antibody (Chemicon). Coverslips were exposed to 1 mg/ml anti-YY1 antibody for 1 h and washed three times in PBS, followed by a 40-min incubation with 1 μg/ml fluorescein isothiocyanate-conjugated anti-rabbit antibody (Chemicon). Coverslips were exposed to 1 μg/ml DAPI (Siga) in PBS for 1 min, mounted on slides with Vectashield mounting solution (Vector Laboratories, Burlingame, CA), and photographed on an Olympus BX510 fluorescence microscope.

**RESULTS**

**Localization of the Protein Binding Site in the Vaccinia I1L Promoter**—We previously described the identification of a late promoter binding activity in extracts from uninfected HeLa cells (7). Prior nuclease footprinting experiments indicated that the protein bound the I1L promoter in the region surrounding the transcriptional start site (7). To delineate the sequences in the I1L promoter required for interaction with the cellular protein, a series of promoter mutants were constructed in which blocks of five nucleotides on the nontemplate strand were replaced with C residues spanning nucleotides −30 to +11, relative to the 5′-A in the conserved TAAAT motif at the start site for transcription (Fig. 1). All of the promoter variants were capable of binding the protein with the exception of mutant 7, which has substitutions in nucleotides +2 to +6 overlapping the TAAAT motif at the start site for transcription. The TAAAT motif is highly conserved in vaccinia late promoters and is usually located at the initiation codon of late gene open reading frames (16).

The sequence immediately surrounding the nucleotides +2 to +6 of the I1L promoter was searched for recognition motifs for known transcription factors using the TRANSFAC program (17) accessed from the WWW site at GFB-Brauschweig, Germany. The search revealed a core recognition sequence for transcription factor YY1 (CCATT) on the template strand. Two properties of YY1 supported its identity as the late promoter-binding protein. First, SDS-polyacrylamide gel analysis of column fractions generated in the purification of the late promoter-binding protein often detected a co-purifying 65-kDa polypeptide on SDS-polyacrylamide gels (data not shown). YY1 was described previously as having an anomalous mobility on SDS-polyacrylamide gels, consistent with a 65-kDa polypeptide although it is 414 amino acids in length (9). Also, it was previously reported that human YY1 binds Ni²⁺, presumably through the 11 consecutive histidine residues in its amino acid sequence (18).

**YY1 Antibodies Recognize the Late Promoter-Binding Protein**—To investigate whether the late promoter-binding activity was YY1, late promoter-binding protein was tested for immunoreactivity with anti-YY1 antibodies. Nickel-purified promoter-binding protein was chromatographed on a phosphocellulose column that was developed with a NaCl gradient. Eluting fractions were assayed for I1L promoter binding and YY1 by Western blotting with anti-YY1 antibody. Both the promoter binding activity and a polypeptide with an apparent mass of 65 kDa recognized by the anti-YY1 antibody eluted with a similar profile peaking in fraction 32 (Fig. 2). Protein from the peak fraction from the phosphocellulose chromatography subsequently was subjected to velocity sedimentation on a glycerol gradient. Again, both the promoter binding activity and the anti-YY1 antibody-reactive 65-kDa polypeptide were observed to co-fractionate, peaking in fraction 20. The sedimentation rate of the promoter-binding activity was essentially identical to that of the 4.46-S hemoglobin sedimentation marker.

The identity of the late promoter-binding protein was confirmed by antibody supershift assays. In electrophoretic mobility shift experiments, the protein produced a characteristic complex with the I1L promoter (Fig. 3). Inclusion of anti-YY1 antibody in the binding reaction resulted in a nearly quantitative shift of the protein-DNA complex to a more slowly migrating complex. Antibody alone produced no complex with DNA. Essentially identical results were obtained with the vaccinia H1L promoter in which the protein-DNA complex was observed to shift further in the gel when anti-YY1 was included in the binding reaction. These results demonstrate that YY1 is a component of the protein-DNA complexes observed in gel shift experiments with late promoter probes.
A histidine-tagged version of YY1 was expressed in bacteria and purified under native conditions by nickel-agarose chromatography. The resulting protein preparations produced a protein-DNA complex in native gel electrophoresis that had a slightly slower mobility than native promoter binding activity purified from HeLa cell nuclei (Fig. 4). The construct used to express recombinant protein produces an 11-amino acid extension on the native YY1 protein, possibly having a small effect on the mobility of the protein-DNA complex. Binding reactions were conducted in the presence of a 50-fold mass excess of the nonspecific competitor poly(dI-dC) to ensure specificity of interaction. These results are consistent with YY1 being the vaccinia late promoter-binding protein.

**Importance of the YY1 Binding Site for Late Promoter Function**—Replacement of nucleotides +2 to +6 in the I1L promoter was shown above to abrogate binding to YY1. The last three nucleotides of the consensus YY1 core recognition sequence CCATT in the I1L promoter overlaps the conserved TAAAT motif previously shown to be essential for the function of other vaccinia late promoters (19). We sought a mutation that would affect the interaction of YY1 with a late promoter without perturbing the TAAAT motif. An alteration of the YY1 binding site in the Rous sarcoma virus long terminal repeat promoter initiator element corresponding to the 3′-T in the TAAAT motif and the two following G residues resulted in inability to associate with YY1 (20). We therefore elected to replace the two G residues directly downstream of the TAAAT motif with C residues to test for the significance of the YY1 binding site. The double C mutant promoter was found to have almost undetectable binding to YY1 in a gel shift assay (Fig. 5). In addition, competition binding experiments demonstrated that excess wild-type sequence I1L promoter effectively competed with itself for binding to YY1, whereas the mutant promoter had little capacity to compete with the wild-type promoter in binding to YY1. Identical results were obtained with bacterially expressed YY1 (data not shown). The mobility of the YY1-DNA complex is indicated by the arrow.

**Activation of Vaccinia Promoters by YY1**
ase is located 18 nucleotides downstream of the first one and should serve as an adequate replacement. The two promoter-reporter gene plasmids were transfected into HeLa cells that previously were infected with vaccinia virus. Reporter enzyme levels were found to be very high from the wild-type IIL promoter (Fig. 5). Replacement of the two G residues immediately downstream of the TAAAT motif resulted in a promoter with 8 ± 0.8% of wild-type promoter activity. Because the nucleotide replacements disturbed the site at which translation initiation should occur, we examined the β-galactosidase transcripts produced in transfected cells. Primer extension experiments detected β-galactosidase transcripts in RNA isolated from cells transfected with the construct driven by the wild-type promoter construct; lane 1 is from infected cells transfected with the CC promoter mutant construct, and lane 1 is from infected cells not transfected with plasmid. Lane 4 is primer alone. The bracket indicates the cDNAs derived from the population of RNAs arising from the promoter.

We conclude that the two G nucleotides immediately downstream of the TAAAT motif are important for both association with YY1 and promoter activity in vivo, providing a correlation between transcription activation and YY1 binding to the vaccinia promoter.

Intracellular Localization of YY1 after Infection by Vaccinia Virus—YY1 has been documented as being located in the nucleus of cells (22). The transcription of vaccinia late genes occurs in structures termed virosomes that are found exclusively in the cytoplasm of the cell. For a nuclear protein to function in viral late gene transcription, the normal intracellular targeting of the protein must be altered. To address this issue, we examined the fate of YY1 after vaccinia infection by immunofluorescence microscopy. The reactivity of anti-YY1 antibodies with uninfected cells showed YY1 to be mostly nuclear (Fig. 6), as reported previously (22). By 6 h after vaccinia infection, YY1 was distributed throughout the cells in which nuclei often could not be distinguished from the remainder of the cell. The cells had obvious DAPI staining of cytoplasmic structures as evidence that they were indeed infected with virus. The YY1 staining pattern changed further at later times post-infection, in which distinct spherical structures in the cytoplasm became evident by 16 h of infection. These results indicate that infection by vaccinia virus had a profound effect on the intracellular localization of YY1, causing significant quantities of the protein to accumulate in the cytoplasm. The targeting of YY1 to the cytoplasm by vaccinia infection is apparently not because of a general displacement of DNA binding proteins from the nucleus because the immunolocalization of the transcription factor CREB (cyclic AMP response element binding protein) showed that its intracellular distribution was unaffected by vaccinia infection (data not shown).

**DISCUSSION**

Our characterization of the vaccinia IIL promoter highlighted an important sequence at the start site for transcription that implicated the cellular transcription factor YY1 as a possible regulator of vaccinia virus late gene transcription. The cellular protein that targets vaccinia virus late promoters is shown here to react with antibody directed against the transcription factor YY1. Bacterially expressed YY1 interacted specifically with the IIL promoter, and a simple dinucleotide re-
placement in the YY1 binding site downstream of the conserved TAAAT late promoter motif resulted in both elimination of YY1 binding and significant reduction in promoter strength in vivo. These results provide a compelling argument for a role for YY1 in activation of vaccinia virus late gene transcription. YY1 is a zinc finger transcription factor belonging to the kruppel family of proteins (reviewed in Ref. 23). This factor has been described as a regulator of many cellular and viral promoters. YY1 can function as an upstream activator, a repressor, or an initiator element regulator.

YY1 regulates several cellular genes in the capacity of an initiator element-binding protein, i.e. the protein binds to the promoter overlapping the start site for transcription in a manner that positively activates transcription (23). Contacts with the bases in the recognition motif are made asymmetrically and have been proposed to account for the directionality of transcription activated by YY1 (24). The YY1 binding site in the vaccinia I1L promoter is also over the start site for transcription, suggesting that YY1 may be acting as an initiator element-binding protein in the vaccinia system. An important distinction is that known cellular initiator elements have the YY1 recognition motif on the non-template strand, and vaccinia late promoters have the motif on their template strand. The significance of this distinction is unclear; however, it suggests that vaccinia may utilize the protein for transcription in a way that may differ from that which occurs in nuclei.

To our knowledge, YY1 is the first identified cellular protein documented to function in poxvirus transcription. Rosales et al. (25) described a cellular protein required for transcription from vaccinia intermediate promoters; however, its identity has not been reported. A cellular protein that activates late promoters in vitro has been described by Gunasinghe et al. (6), but its identity has not been reported. YY1 would seem to be a particularly suitable choice for adoption by vaccinia virus. Vaccinia has a rather remarkable host range and can replicate in a wide variety, albeit not all, of mammalian cell lines. YY1 has been described as being ubiquitous in terms of tissue distribution and the numerous mammalian cell lines that have been examined (23). Thus the cellular distribution of YY1 would not likely impose a limit on the host range of the virus.

The YY1 binding site in the I1L promoter overlaps the TAAAT motif on its distal side. Our data argue that the TAAAT and YY1 motifs are overlapping but functionally distinct elements of vaccinia late promoters. The importance of the TAAAT motif for late gene transcription is well documented (19). Replacement of any nucleotide in this motif results in an inactive promoter. We have replaced the 5'-A in the TAAAT motif of the I1L promoter and confirmed its importance for transcription in vivo; however, this change has no effect on YY1 binding (7). Thus the three most 5'-nucleotides of the TAAAT motif serves some function other than interaction with YY1. We replaced the two G residues immediately distal to the TAAAT motif and observed a correlation between transcription in vivo and binding to YY1 in vitro. The importance of nucleotides following the TAAAT motif in a natural vaccinia late promoter had not been described previously.

If nucleotides downstream of the TAAAT motif are essential for promoter function, then is predicted that these sequences should be conserved in vaccinia late promoters. The optimal binding sequence for YY1 has been defined as (C/T/g/a)(G/t)(C/t/a)CATN(T/a)/(T/g/c), in which uppercase letters are the preferred nucleotide and lowercase letters are tolerated to a lesser extent (26). In the context of the TAAAT motif of vaccinia late promoters, the optimal YY1 site on the non-template strand would be TAAATGGCG. Only in the first T residue does this sequence deviate from the optimal YY1 site. This exact sequence is found in the I1L promoter. The first G in this sequence is naturally conserved because the vast majority of vaccinia late genes have translation initiation codons at this location. The following G residue is also highly conserved. An examination of the sequences of 51 late genes revealed through the comparison of vaccinia and molluscum contagiosum virus sequences (27) shows that 30 have G at this position, and 15 others have A.

For a cytoplasmic virus such as vaccinia to utilize a cellular protein that is normally nuclear, a redirection of the trafficking of the protein must occur. In agreement with others (22), we observed YY1 to be predominantly nuclear in uninfected cells. After vaccinia infection, YY1 clearly was distributed throughout the entire cell, and at later times, was concentrated in cytoplasmic globules resembling virosomes, large nucleoprotein complexes that are actively replicating DNA and synthesizing late mRNA. Because the translation of all host mRNA is believed to be inhibited shortly after vaccinia infection (28), the redistribution of YY1 likely reflects an alteration in intracellular trafficking of pre-existing protein. Whether this is a passive process of binding of YY1 to the thousands of accumulating DNA binding sites amplified by viral DNA replication in the cytoplasm or an active process induced by the virus is unclear.

It is of interest to point out that poxviruses can be added to the list of DNA viruses that have been shown to require YY1 as a regulator of transcription. Herpesviruses (29, 30), papillomaviruses (31, 32), polyomaviruses (33), adenoviruses (34), parvoviruses (9, 35), and retroviruses (20, 36) use YY1 in one of its various capacities as a transcriptional regulator. Inclusion of poxviruses in this group indicates that virtually all of the major DNA virus groups require YY1 function. The widespread utilization of this protein underscores its fundamental importance in the transcription process.

Acknowledgments—We are grateful to Tom Shenk for the pHIS YY1 plasmid and to Jonathan LeBowitz and John Maga for advice on immunofluorescence and image processing.

REFERENCES

1. Moss, B. (1996) in Fields Virology (Fields, B. N., Knipe, D. M. & Howley, P. M., eds), pp. 2637–2671, Lippincott-Raven Publishers, Philadelphia.
2. Moss, B. (1994) in Transcription Mechanisms and Regulation (Conaway, R. C. & Conaway, J. W., eds), pp. 185–205, Raven Press, New York.
3. Keck, J. G., Baldick, C. J. J. & Moss, B. (1990) Cell 61, 801–809.
4. Kovacs, G. R. & Moss, B. (1996) J. Virol. 70, 6796–6802.
5. Wright, C. F. & Cornea, L. M. (1993) J. Virol. 67, 7264–7270.
6. Gunasinghe, S. K., Hubbs, A. E. & Wright, C. E. (1998) J. Biol. Chem. 273, 27524–27530.
7. Zhu, M., Moore, T. & Broyles, S. S. (1998) J. Virol. 72, 3893–3899.
8. Dignam, J. D. (1990) Methods Enzymol. 182, 194–203.
9. Shi, Y., Seto, E., Chang, L. S. & Shenk, T. (1991) Cell 67, 377–388.
10. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, pp. 10.51–10.53, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
11. Broyles, S. S., Yuen, L., Shuman, S. & Moss, B. (1988) J. Virol. 62, 10754–10760.
12. Harlow, E. & Lane, D. (1988) Antibodies: A Laboratory Manual, pp. 386–387, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
13. Mikaelian, I. & Sergeant, A. (1992) Nucleic Acids Res. 20, 328.
14. Miller, J. (1972) in Experiments in Molecular Genetics, pp. 352–355, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
15. Bethel, C., Van Meir, E., ten Heggelder-Bordier, B. & Wittek, R. (1987) Cell 56, 153–162.
16. Roschl, J., Earl, P. L., Weir, J. P. & Moss, B. (1986) J. Virol. 60, 436–439.
17. Heinemeyer, T., Chen, X., Karas, H., Kel, A. E., Kel, O. V., Liebich, I., Meidhart, T., Reuter, I., Schacherer, P. & Wingender, E. (1999) Nucleic Acids Res. 27, 318–322.
18. Hu, X., Wolfe, E. J., Weisberg, A. S., Carroll, L. J. & Moss, B. (1998) J. Virol. 72, 104–112.
19. Davison, A. J. & Moss, B. (1989) J. Biol. Chem. 20, 771–784.
20. Mohley, C. N. & Sealy, L. (1998) J. Virol. 72, 6592–6601.
21. Schwer, B., Visca, P., Vos, J. C. & Stunnenberg, H. G. (1987) Cell 50, 163–169.
22. Austen, M., Luscher, B. & Luscher-Fizla, J. M. (1997) J. Biol. Chem. 272, 1709–1717.

(X. Liu and S. S. Broyles, unpublished results.)
Activation of Vaccinia Promoters by YY1

23. Shi, Y., Lee, J.-S. & Galvin, K. M. (1997) Biochim. Biophys. Acta 1332, F49–F60
24. Houbaviy, H. B., Usheva, A., Shenk, T. & Burley, S. K. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13577–13582
25. Rosales, R., Sutter, G. & Moss, B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3794–3798
26. Hyde-DeRuyscher, R. P., Jennings, E. & Shenk, T. (1995) Nucleic Acids Res. 23, 4457–4465
27. Senkevich, T. G., Koonin, E. V., Bagert, J. J., Darai, G. & Moss, B. (1997) Virology 233, 19–42
28. Moss, B. (1968) J. Virol. 2, 1028–1037
29. Chen, S., Mills, L., Perry, P., Riddle, S., Wobig, R., Lown, R. & Millette, R. I. (1992) J. Virol. 66, 4304–4314
30. Bell, A., Skinner, J., Kirby, H. & Rickinson, A. (1998) Virology 252, 149–161
31. Bauknecht, T., Angel, P., Royer, H.-D. & zur Hausen, H. H. (1992) EMBO J. 11, 4607–4617
32. Pajunk, H. S., May, C., Pfister, H. & Fuchs, P. G. (1997) J. Gen. Virol. 78, 3287–3295
33. Martelli, F., Iacobini, C., Caruso, M. & Felsani, A. (1996) J. Virol. 70, 1433–1438
34. Zeck, C., Iselt, A. & Doerfler, W. (1993) J. Virol. 67, 682–698
35. Momoeda, M., Kawas, M., Janes, S. M., Miyamura, K., Young, N. S. & Kajigaya, S. (1994) J. Virol. 68, 7159–7168
36. Knossl, M., Lower, R. & Lower, J. (1999) J. Virol. 73, 1254–1261