Antiviral Activity of Distamycin A against Vaccinia Virus Is the Result of Inhibition of Postreplicative mRNA Synthesis

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Distamycin A has been described as an inhibitor of the cellular pathogenesis of vaccinia virus in culture. Distamycin is an antibiotic that specifically targets the minor groove of DNA. We show here that distamycin is a potent inhibitor of vaccinia virus replication. Pulse-labeling experiments showed that most major late proteins failed to accumulate in the presence of the antibiotic. We characterized the effect of distamycin on vaccinia virus nucleic acid biosynthesis with the goal of determining the inhibitor's target. Early gene transcription was unaffected. DNA synthesis proceeded at normal rates, but DNA accumulated in large masses in the cytoplasm with no evidence of virion assembly. Transcription from the intermediate class promoter for the 11L gene was partially reduced by distamycin; however, transcription from the intermediate promoters for the three late transcription factor genes was severely inhibited. The accumulation of the late transcripts for the viral F17R and A10L genes also was severely impaired and was shown to be a direct inhibition of late promoter activity. These results indicate that inhibition of postreplicative intermediate and late transcription is the basis for inhibition of vaccinia virus by distamycin and indicate that DNA minor-groove ligands hold promise for effective anti-poxvirus drugs.

Distamycin A is an antibiotic that reduces the pathogenic effects of vaccinia virus. Distamycin blocks virus-induced cell syncytium formation (22) and inhibits the visually observed cytopathic effects of the virus in cultured cells (16). Distamycin targets the minor groove of DNA (10), preferentially binding DNA sequences that are five consecutive A-T pairs, with affinity that varies with the particular sequence (1). The base composition of the DNA genome of vaccinia virus is 66% A-T, with transcriptional promoter regions being close to 90% A-T, making them ideal targets for the antibiotic.

Vaccinia virus replicates its genome and assembles progeny virions exclusively in the cytoplasm of the infected cell. The processes of DNA synthesis and transcription of the three different classes of genes are coordinated in a sequential manner (reviewed in reference 4). Upon entry of the cell, viral early gene transcription begins immediately. The early gene products include factors participating in DNA synthesis that begins about an hour after infection. As DNA synthesis initiates, early gene transcription ceases and intermediate gene transcription ensues. Shortly thereafter, late gene transcription begins and intermediate gene transcription wanes. The virus-encoded proteins required for the transcription of each gene class are products of the preceding class in a gene expression cascade. The promoters for vaccinia virus early, intermediate, and late genes have A-T-rich motifs whose interaction with transcription factors potentially could be affected by a DNA minor-groove ligand. The early promoter element is targeted by the early transcription factor (6). We have shown that distamycin impairs DNA binding by the early transcription factor in vitro (S. S. Broyles, submitted for publication), thus predicting one basis for inhibition of virus replication. Intermediate promoters consist of an upstream element and an initiator-like element at the transcription start site (2). The initiator element of the intermediate 11L promoter may be a target for the cellular transcription factor YY1 (5). YY1 is a zinc finger transcription factor that does not contact the minor groove of DNA (12). The protein that targets the upstream intermediate element is unknown. Late promoters also have upstream and initiator elements. Heterogeneous nuclear riboproteins A2/B1 and RBM3 have been shown to stimulate vaccinia virus late transcription in vitro and target oligo(T) tracts in DNA (23) previously shown to behave as an upstream element in a late promoter (9). The role of the late promoter initiator element is not known. DNA synthesis is another potential target of distamycin. Whether vaccinia virus has an origin of replication that is targeted by origin-binding proteins is unclear.

In this study, we characterized the effect of distamycin on vaccinia virus nucleic acid biosynthesis. We established that distamycin is an inhibitor of virus multiplication and followed the synthesis of viral DNA and the three classes of mRNA to determine the target of the drug.

Antiviral effects of distamycin A against vaccinia virus. The effect of distamycin A on the replication of vaccinia virus was assessed initially in a virus plaque assay. Monolayer cultures of BSC40 cells were infected with approximately 50 PFU of virus for 48 h in the presence of increasing concentrations of the drug and subsequently stained with crystal violet to visualize remaining adherent cells. Distamycin was applied to the cells immediately after the virus, as it was in all subsequent experiments. High concentrations of the drug (50 and 100 μM) resulted in complete disappearance of plaques (Fig. 1A). Intermediate concentrations of the drug (1 to 20 μM) reduced the diameter of plaques but did not significantly affect the number of visible plaques. We followed the course of infection for up to 6 days, when the antibiotic begins to show cytotoxic effects, and observed no sign of virus plaques.
The effects of distamycin on vaccinia virus multiplication were evaluated further in a single-step growth experiment. BSC40 cells were infected with vaccinia virus for 24 h in the presence of various drug concentrations, and the virus yield was determined in a subsequent plaque assay. High concentrations of distamycin (>50 μM) reduced the virus yield by approximately 2 log units relative to infection in the absence of the drug (Fig. 1B). Virtually identical results were obtained when infection was allowed to proceed for 24 h (data not shown). For all further experiments, the distamycin concentration was 100 μM.

The effect of distamycin on the global vaccinia virus gene expression profile was assessed by monitoring protein synthesis during the course of infection. BSC40 cells were infected with 10 PFU of virus per cell and pulse-labeled for 30 min with [35S]methionine at intervals from 2 to 24 h after infection. In the absence of the drug, shutoff of host protein synthesis was evident within a few hours, early proteins were made within the first few hours and declined, and major proteins species appeared at 4 h after infection (Fig. 2). In the presence of distamycin, host protein shutoff was also evident and early viral proteins also appeared in the first few hours of infection. In the distamycin-treated cells, the early proteins appeared to persist during the course of the experiment and the major late proteins failed to appear. This experiment suggests that early events in the infectious cycle are unaffected; however, late protein synthesis appears to be defective. Interestingly, one major protein with the mobility of a 35-kDa protein appeared late in the presence of distamycin.

Effect of distamycin on viral nucleic acid metabolism. Because distamycin is a DNA minor-groove ligand, it is reasonable to expect it to exert inhibitory effects on DNA replication and/or viral gene transcription. To determine the effect of distamycin on early gene transcription, BSC40 cells were infected with vaccinia virus for 1 and 2 h. Total RNA was isolated and analyzed by Northern blotting with a probe for the C11L (growth factor) gene, which is an early class gene (21). The C11L gene probe detected the expected 600-base transcript in RNA from cells infected for 1 h that increased further at 2 h (Fig. 3A). Inclusion of distamycin in the culture medium did not affect transcript accumulation at either of the two time points. We concluded that distamycin does not affect vaccinia virus early gene transcription significantly in vivo.

We next determined the effect of distamycin on replication of the vaccinia virus genome. Total DNA was extracted from cells at various times after infection, and viral DNA was identified by slot blot hybridization with radiolabeled vaccinia virus genomic DNA as the probe. As expected, viral DNA was detectable by 2 h after infection and continued to accumulate through 8 h (Fig. 3B). Inclusion of distamycin in the culture
medium affected neither the onset of DNA replication nor the level of accumulation of viral DNA through 8 h postinfection. We concluded that vaccinia virus DNA replication is not affected by distamycin. Because DNA replication requires the expression of multiple early gene products, the ability to synthesize DNA supports the conclusion that distamycin does not affect early gene transcription.

We next examined the effect of distamycin on intermediate and late gene transcription. Intermediate and late transcripts are moderately heterogeneous at their 5' ends because of apparent slippage of the RNA polymerase on the TTT in the initiator motif on the template DNA, producing mRNAs with 15 to 35 non-template-encoded A residues (3, 20). They are also extremely heterogeneous at their 3' ends because the RNA polymerase terminates randomly (for example, see reference 24). We therefore used primer extension to detect the 5' ends of intermediate and late mRNAs from virus-infected cells. We have demonstrated that the I1L promoter belongs to the intermediate class and confirmed that the F17R promoter

is a late class gene (X. Liu and S. S. Broyles, unpublished observations). Primers were designed to hybridize 120 nucleotides downstream of the F17R transcription initiation site and 150 nucleotides downstream of the I1L transcription initiation site, respectively. Simultaneous analysis of the primer extension products from both mRNAs showed that the I1L message was reduced by 22% in RNA derived from cells infected with virus in the presence of distamycin relative to its absence, as determined with a phosphorimager (Fig. 2C). The level of F17R mRNA appeared to be more dramatically affected. Primer extension products corresponding to the F17R mRNA appeared to be absent in RNA from distamycin-treated cells.

The loss of late transcription coupled with a partial inhibition of transcription from an intermediate promoter could be explained by inhibition of transcription of late factor genes that are intermediate genes. We therefore tested the effect of distamycin on the transcription of the intermediate class A1L, A2L, and G8R promoters that encode late transcription factors (14). The activity of these promoters is only a fraction of that of the I1L intermediate promoter (Liu and Broyles, unpublished), making detection of the heterogeneous primer extension products problematic. As an alternative, a reporter gene approach was used to monitor the activity of the late transcription factor promoters. We linked each of the three promoters to the lacZ reporter gene and transfected each into HeLa cells previously infected with vaccinia virus. Reporter gene expression from each of the three promoters was inhibited by distamycin. Reporter activity of the A1L, A2L, and G8R promoters was reduced by greater than 90% in the presence of distamycin relative to its absence (Fig. 4). We conclude that the transcription of some intermediate genes, as typified by the late factor genes, is highly sensitive to the effects of distamycin. The re-
porter activity of the I1L reporter construct retained almost 60% of its activity in the presence of distamycin, in agreement with the primer extension results.

The effect of distamycin on late gene transcription was assessed in more detail by primer extension with primers designed to hybridize to the F17R and A10L (p4a gene) mRNAs. We have confirmed that the promoters for both genes belong to the late class (Liu and Broyles, unpublished). RNA was isolated at 6, 8, and 24 h after infection with vaccinia virus and analyzed by primer extension with 30-mer primers designed to initiate extension 50 nucleotides upstream of the initiation site in each promoter. Primer extension on RNA from cells infected with virus in the absence of DNA synthesis produced heterogeneous products of the expected length for both mRNAs (Fig. 5). The range of primer extension product lengths for both mRNAs was about 60 to 85 nucleotides, consistent with 50 nucleotides of mRNA plus the length of the non-template-encoded oligo(A) tract previously described for late mRNAs (3, 20). Primer extension products also included longer products that we call readthrough products, although we do not know whether they reflect transcripts originating upstream of each promoter or are the result of primer hybridization to other transcripts. Primer extension on RNA from cells infected with virus in the presence of distamycin showed virtually no products corresponding to either promoter, nor did they produce appreciable signal from the readthrough RNA (Fig. 5). These results demonstrate that late transcription is extremely sensitive to distamycin.

The results presented above raised the question of whether distamycin has a direct effect on late gene transcription or only an indirect effect of impaired expression of the late transcription factors. To address this issue, we circumvented the late transcription requirement for intermediate transcription by expression of the three late transcription factors with a T7 RNA polymerase-directed system (14, 15). HeLa cells were infected with a vaccinia virus expressing the bacteriophage T7 RNA polymerase and transfected with a β-galactosidase reporter plasmid driven by the A10L late vaccinia virus promoter together with plasmids encoding the A1L, A2L, and G8R genes driven by a T7 promoter. Expression of the T7 RNA polymerase is driven by a tandem early-late promoter and therefore proceeds in the absence of DNA synthesis. This protocol allows late promoters to be activated in the absence of intermediate transcription. In the reporter gene assays, distamycin alone was a potent inhibitor of the A10L late promoter, as was hydroxyurea (HU), an inhibitor of DNA synthesis (Fig. 6). The activity of the A10L promoter in the presence of HU was restored by coexpression of the three late transcription factors, which also was inhibited by distamycin. A reporter construct driven by a T7 promoter retained more than 60% of its activity in the presence of distamycin, indicating that inhibition of the T7 RNA polymerase was not the basis for inhibition of late transcription. These results indicate that late transcription is inhibited directly by distamycin, similar to that observed for the intermediate transcription described above.

To further characterize the defects in viral replication induced by distamycin, we examined virus-infected cells by transmission electron microscopy. Vaccinia virus morphogenesis proceeds through a series of discrete structures that are readily distinguished by electron microscopy (8). Membrane crescents initially form to envelop nascent genomes to assemble spherical immature...
particles that later condense to form oblate mature virions. Cells infected with vaccinia virus in the absence of the drug for 24 h displayed all of these morphogenetic species (data not shown). Cells infected with vaccinia virus in the presence of distamycin showed no evidence of the virion assembly intermediates normally found in virus-infected cells. Instead, these cells contained large cytoplasmic structures referred to as viroplasm bodies. These viroplasm bodies appear to exclude cytoplasmic organelles, being bordered by a membrane at its periphery in some areas but not in others. We inferred that these structures contain DNA because fluorescence microscopy of cells infected with vaccinia virus in the presence of distamycin detected cytoplasmic staining with the DNA dye 4',6'-diamidino-2-phenylindole (data not shown). We concluded that distamycin allows the accumulation of high levels of viral DNA but has a profound effect on assembly of progeny virions.

Distamycin A is shown here to be a potent inhibitor of vaccinia virus replication. Infection at the level of a plaque assay was essentially completely negated by high concentrations of distamycin. Virus yield was reduced by about 2 orders of magnitude. Electron microscopy showed no evidence of virus assembly intermediates beyond large masses of DNA in the cytoplasm of antibiotic-treated cells.

Our characterization of viral nucleic acid synthesis in the presence of distamycin indicated that vaccinia virus intermediate and late gene transcription is inhibited by the antibiotic. Early gene transcription and DNA synthesis appeared to proceed normally in the presence of the antibiotic. Transcription from the intermediate III promoter was partially inhibited, whereas transcription from the intermediate A1L, A2L, and G8R promoters was almost totally inactivated by distamycin. The reason for differential sensitivity to distamycin among intermediate promoters is unclear. We noted that an abundant 35-kDa protein appeared to be resistant to the effects of distamycin. The predicted mass of the I11 protein is 35 kDa (13). The protein that targets the upstream element in intermediate promoters has not been identified, and no protein interacting with the minor groove of DNA has been implicated in vaccinia virus intermediate transcription. The phenotype of vaccinia virus in the presence of distamycin is one that closely recapitulates the phenotype of some conditional lethal mutants with lesions in RNA polymerase subunit genes (11). RNA polymerase mutants are defective for late gene transcription but normal for early gene transcription. While the characterization of these mutants was reported prior to the discovery of the intermediate class of vaccinia virus genes, RNA polymerase mutants are likely defective for intermediate transcription as well. The RNA polymerase mutants also produce large viroplasm masses of DNA in the cytoplasm and fail to produce virion assembly intermediates. These mutants also are defective for processing of genome DNA concatemers into unit lengths (17), which probably explains why the DNA remains associated in large aggregates.

The combined effects of inhibition of late transcription factor synthesis and inhibition of late transcription by distamycin no doubt constitute a particularly potent means by which to block vaccinia virus late gene expression. The results presented here indicate that DNA minor-groove ligands may hold promise for the development of new anti-poxvirus drugs. While distamycin itself is too toxic for therapeutic purposes, other minor-groove ligands with specificity for vaccinia virus promoters may be developed. DNA minor-groove ligands may also be useful for investigations into which inhibiting postreplicative mRNA synthesis is desired without disturbing DNA replication.

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REFERENCES


