An Isatin-β-thiosemicarbazone-resistant Vaccinia Virus Containing a Mutation in the Second Largest Subunit of the Viral RNA Polymerase Is Defective in Transcription Elongation*

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The vaccinia virus RNA polymerase is a multi-subunit enzyme that contains eight subunits in the postreplicative form. A prior study of a virus called IBT™, which contains a mutation in the A24 gene encoding the RPO132 subunit of the RNA polymerase, demonstrated that the mutation results in resistance to the anti-poxvirus drug isatin-β-thiosemicarbazone (IBT). In this study, we utilized an in vitro transcription elongation assay to determine the effect of this mutation on transcription elongation. Both wild type and IBT™ polymerase complexes were studied with regard to their ability to pause during elongation, their stability in a paused state, their ability to release transcripts, and their elongation rate. We have determined that the IBT™ complex is specifically defective in elongation compared with the WT complex, pausing longer and more frequently than the WT complex. We have built a homology model of the RPO132 subunit with the yeast subunit RPO132 to propose a structural mechanism for this elongation defect.

Vaccinia virus, the prototypic Orthopoxvirus, contains ~200 kb of double-stranded linear DNA. Vaccinia virus replicates in the cytoplasm of host cells and encodes its own multi-subunit RNA polymerase and associated transcription machinery, making it a convenient model for transcriptional studies (1). There are three temporal stages of transcription after a vaccinia virus infection: early; intermediate; and late. For the purposes of this study, the latter two stages are referred to collectively as postreplicative.

Early viral transcription has been well characterized, whereas less is known regarding postreplicative transcription. Each stage of transcription is distinguished from the other stages by the recognition of different promoter classes and the utilization of distinct transcription factors. Both early and postreplicative transcription complexes have eight polymerase subunits in common. Of these, RPO147 and RPO132, the products of the J6R and A24R genes, respectively, have some sequence similarity to the two largest subunits of the yeast pol II RNA polymerase. Another subunit, RPO30, which is the product of the E4L gene, has some sequence similarity to the yeast-positive transcription elongation factor TFIIS (2, 3). The early vaccinia RNA polymerase contains one additional subunit, the product of the H4L gene, Rap94, which is not present in postreplicative transcription complexes (4–8). Rap94 appears to be involved in the recognition of promoter classes, because polymerase complexes that contain Rap94 transcribe only early and not late genes and complexes that do not contain Rap94 transcribe only late and not early genes (6, 7, 9, 10).

Several factors distinguish early transcription elongation and termination from postreplicative transcription elongation and termination. The elongation complex of early genes is fairly stable in the presence of salt and sarkosyl, and one early transcription elongation factor, nucleoside triphosphate phosphohydrolase I, has been identified (11–13). Early transcripts contain an intrinsic termination signal, U5NU, 30–50 nucleotides (nt) upstream of the mRNA 3′ end (14–16). Nucleoside triphosphate phosphohydrolase I and the virus-coded mRNA-capping enzyme are both required for early gene transcription termination, which results in the production of transcripts from any given early gene with 3′ ends that are uniform in length (15–18). Postreplicative genes do not respond to the early termination signal and lack any other known termination signal. Their transcripts from any given intermediate or late gene are heterogeneous in size at their 3′ ends (19, 20). At least three vaccinia virus-encoded proteins are believed to be involved in the regulation of postreplicative transcription elongation and termination. A postreplicative transcript release factor, A18, has been identified in vivo and characterized in vitro (21, 22), and two putative postreplicative transcription elongation factors, G2 and J3, have been identified through in vivo experiments (23, 24). To confirm the roles of G2 and J3 as elongation factors, an in vitro elongation assay is essential but first the assay must be utilized to evaluate the basic biochemistry of postreplicative transcription with regard to elongation rate and stability in the presence of salt, sarkosyl, and heparin.

The anti-poxvirus drug isatin-β-thiosemicarbazone (IBT) has been useful in vivo for studying postinitiation events in postreplicative gene transcription. IBT has no effect on early transcription, but during postreplicative transcription, IBT causes read-through transcription resulting in the production of longer than wild type (WT) length transcripts, suggesting that the drug affects elongation or termination. Read-through transcription from converging promoters leads to the production of excess double-stranded RNA. This causes the activation of the cellular 2-5A pathway and induction of RNase L activity, which inhibits viral growth (25). Both IBT-dependent and IBT-resistant mutants can be isolated, and these mutants provide insight into genes that regulate postreplicative transcription (26). Mutants that are dependent on IBT map to genes that seem to encode elongation factors, specifically G2 and J3 (23). IBT-dependent null mutations in these genes have been shown in vivo to produce shorter than WT transcripts in the absence of IBT.
(23, 24). Therefore, in the absence of IBT, G2 and J3 mutants that are IBT-dependent make postreplicative transcripts that are too short to encode all of the proteins needed to maintain viability of the virus. The addition of IBT to these IBT-dependent viruses causes an increase in the length of postreplicative transcripts that restores viability of the virus.

IBT resistance presents a more complicated scenario. The target and precise mechanism of action of IBT are unknown, but IBT resistance could theoretically result from a mutation in a gene needed to activate the drug, a mutation that abrogates the binding of the drug, or a mutation that compensates for the effect of the drug. It has been proposed previously that IBT resistance represents an intermediate phenotype between IBT sensitivity and IBT dependence (27). Specifically, IBT-resistant viruses may produce postreplicative transcripts that are shorter than those produced by an IBT-sensitive virus but not as short as those produced by an IBT-dependent virus. In the absence of IBT, the majority of transcripts would be long enough to encode functional proteins, and in the presence of IBT, the majority of transcripts would not be so long as to form a significant amount of double-stranded RNA. IBT-resistant mutants have been isolated that map to the J3R and A24R genes. The IBT-resistant virus with a mutation in the A24R gene has not been characterized with regard to transcript length in vivo or in vitro (28). IBT-resistant viruses with a J3 mutation have recently been studied in vivo with regard to transcript length, and these transcripts do not appear to be measurably different in size from those produced by WT or IBT-sensitive viruses in the absence of IBT or from transcripts produced by IBT-dependent viruses in the presence of IBT (23).

It is possible that subtle size differences in transcript length from IBT-resistant viruses in the presence and absence of IBT were difficult to detect in vivo because of the fact that postreplicative vaccinia virus transcripts are heterogeneous in size.

To address the mechanism of IBT action, we have modified an in vitro transcript release assay utilized to identify the A18 protein as a transcript release factor and used it to examine postreplicative transcription elongation of the IBT-resistant virus, IBT(90). The IBT(90) virus contains a T to C substitution at nt 1384 in the A24 gene, which encodes RPO132, the second largest subunit of the RNA polymerase. Both IBT(90) and WT vaccinia viruses were analyzed with regard to pausing, elongation, and stability of the polymerase complexes in postreplicative transcription. Results demonstrate that in vitro IBT(90) is defective in transcription elongation.

**EXPERIMENTAL PROCEDURES**

**Terminology**—All of the vaccinia virus strain WR genes discussed in this paper are referred to by their location in a HindIII restriction digest. A new nomenclature based on sequential numbering of the genes has been implemented. These new names are mentioned in the text where appropriate.

**Cell Culture and Infected Cell Extracts for Transcription**—A549 cells were propagated in 1× Dulbecco’s modified Eagle’s medium with 10% bovine calf serum (Hyclone) at 37 °C with 5% CO2. Confluent 100-mm dishes of A549 cells were infected with either WT vaccinia virus strain WR (29) or IBT(90) virus (28) at a multiplicity of infection of 15 at 37 °C and then incubated in medium containing 10 mM hydroxyurea, an inhibitor of postreplicative transcription elongation of the IBT-resistant virus with a mutation in the A24R gene has not been characterized with regard to transcript length in vitro. Results demonstrate that in vitro IBT(90) is defective in transcription elongation.

**In Vivo Transcription Elongation Assay**—During the pulse phase of the elongation assay, transcription initiation complexes were formed at the viral G8 promoter by combining 15 μl of infected cell extracts with 5 μl of cold 5’-G-less DNA template and 5 μl of transcripts for a final volume of 25 μl containing 5 mM MgCl2, 25 mM HEPES, pH 7.4, 1.6 mM dithiothreitol, 80 mM KOAc, 1 mM ATP, 1 mM UTP, 1 mM CTP, 200 μM 3’-O-methyl-GTP, and 6 μl of [α-32P]CTP (3000 Ci/mmol stock, PerkinElmer Life Sciences). Complexes were incubated at 30 °C for 20 min to allow elongation to proceed to the end of the 37-nt G-less cassette. Complexes were isolated by binding the paramagnetic bead-bound templates to a magnet and were then washed 3× in 1–1.5 reaction volumes of low salt wash buffer containing 5 mM MgCl2, 25 mM HEPES, pH 7.4, 1.6 mM dithiothreitol, 80 mM KOAc, 200 μg/μl bovine serum albumin, and 7.5% glycerol. Complexes were resuspended in transcription buffer containing 5 mM MgCl2, 25 mM HEPES, pH 7.4, 1.6 mM dithiothreitol, 80 mM KOAc, 200 μg/ml bovine serum albumin, and 7.5% glycerol. Complexes were resuspended in transcription buffer containing 5 mM MgCl2, 25 mM HEPES, pH 7.4, 1.6 mM dithiothreitol, 80 mM KOAc, 200 μg/ml bovine serum albumin, and 7.5% glycerol. Complexes were resuspended in transcription buffer containing 5 mM MgCl2, 25 mM HEPES, pH 7.4, 1.6 mM dithiothreitol, 80 mM KOAc, 200 μg/ml bovine serum albumin, and 7.5% glycerol. Complexes were resuspended in transcription buffer containing 5 mM MgCl2, 25 mM HEPES, pH 7.4, 1.6 mM dithiothreitol, 80 mM KOAc, 200 μg/ml bovine serum albumin, and 7.5% glycerol.

**Sequencing**—The IBT(90) A24R gene (VACWR144) was PCR-amplified using primers upstream and downstream of the open reading frame. This PCR product was submitted to the University of Florida Interdisciplinary Center for Biotechnology Research for sequencing. Sequencing reads were assembled using the Wisconsin package, version 10.3 (Accelrys Inc., San Diego, CA).
and, where applicable, exposed to a PhosphorImager screen (Amersham Biosciences) that was analyzed using a Storm PhosphorImager and the ImageQuant (Amersham Biosciences) program.

**Modeling**—A homology model of the RPO132 protein was constructed using ClustalW, Swiss-PdbViewer, version 3.7, and the Swiss-Model homology modeling server (swissmodel.expasy.org) (31, 32). An amino acid sequence alignment of RPO132 and *Saccharomyces cerevisiae* rpβ2 (Protein Data Bank codes 16th and In1k) was constructed using ClustalW and used by Swiss-PdbViewer to generate a structural alignment. The structural alignment was edited by hand and submitted to the SwissModel as an optimize request for construction of a homology model. The Swiss-PdbViewer was used to export views of the model as Mega-Pov scenes that were rendered using POV-Ray (www.pov-ray.org/), version 3.5, for linux.

**RESULTS**

To investigate vaccinia virus postreplicative transcription elongation, we modified an *in vitro* release assay that had previously been used to investigate postreplicative termination (22). In this system, a linear DNA template that is biotinylated at one or both ends is bound to streptavidin-coated paramagnetic beads to form a transcription template (Fig. 1). The templates used in this study, pG8GU and pfeU1, are both under the control of the vaccinia virus intermediate G8 promoter followed by a 37-nt G-less cassette in the non-template strand. The DNA template is incubated with vaccinia virus-infected cytoplasmic extracts and with transcription buffer plus ATP, UTP, [γ-32P]CTP, and 3'-O-methyl-GTP to initiate a pulse reaction. The incorporation of the 3'-O-methyl-GTP at the end of the 37-nt G-less cassette causes the polymerase complex to arrest at that point. The template is then isolated by binding the paramagnetic beads to a magnet, and the polymerase complex is washed in transcription buffer to remove any unassociated factors. The bead-bound complexes are resuspended in transcription buffer, and transcription elongation resumes in the chase reaction when NTPs are added and the intrinsic cleavage activity of the RNA polymerase cleaves the 3'-O-methyl-GTP residue from the arrested complex (33). This cleavage activity was demonstrated for the early polymerase complex, and the ability of the postreplicative complex to recover from incorporation of a 3'-O-methyl-GTP residue indicates that this cleavage activity is also present at intermediate and late times. The two DNA templates used in this study were designed specifically to evaluate the pausing of the polymerase in the chase reaction. Following the 37-nt G-less cassette, the pG8GU template contains a 40-nt T-less cassette followed by a stretch of 9 T residues in the non-template strand, which is known to induce the pausing of the polymerase complex at limiting UTP chase concentrations (Fig. 1A). This 17-nt poly(T) site is followed by 23 nt of the remaining E1L/F17R coding region. The pfeU1 template contains 603 nt of the vaccinia virus E1L and F17R coding regions downstream of the 37-nt G-less cassette (Fig. 1B). A portion of the E1L/F17R coding region was removed to shift a poly(T) site (15/17 nt are Ts) closer to the G8 promoter on this template. This 17-nt poly(T) site is followed by 233 nt of the remaining E1L/F17R genes and 3230 nt of plasmid DNA.

To test the *in vitro* elongation system in a pausing assay, we initiated a pulse reaction on the pG8GU template using WT vaccinia virus extracts (Fig. 2A, lane 1). The pulse reaction yields heterogeneous transcripts ranging in size from ~50 to 75 nt due to the presence of a 5'-poly(A) “head” averaging 30 nt in size that is added to postreplicative transcripts as a result of the RNA polymerase complex stuttering at the promoter (34–38). Transcription complexes were chased in the presence of 1 mM ATP, CTP, and GTP and varying amounts of UTP for 2 or 20 min, and bead-bound transcripts (B) were separated from the released transcripts (R). The percentages of full-length and bound transcripts were calculated and graphed (Fig. 2, B and C). In the absence of UTP, the majority of the transcripts are paused at the poly(T) site in both the 2- and 20-min chase reactions (Fig. 2A, lanes 2 and 20, and B). As increasing amounts of UTP are added, there is an increase in the amount of full-length products produced. At 1 mM UTP, little pausing seems to occur because the majority of transcripts reach full length only after a 2-min chase (Fig. 2, A, lane 18, and B). As
noted previously, the amount of transcript release increases with longer chase times and there is a large increase in the percentage of released transcripts after the 20-min chase compared with the 2-min chase (Fig. 2C) (22). These data indicate that the poly(T) site on the pG8GU template does act as a major pause site for the polymerase complex when the complex is starved for UTP in the chase reaction. Therefore, this template serves as an excellent tool for evaluating the ability of the vaccinia virus RNA polymerase complex to elongate under various conditions.

To evaluate transcription elongation on the pfeU1 template, a pulse reaction was initiated on pfeU1 with WT-infected cell extracts. The polymerase complex was washed and chased in the presence of 1 mM ATP, CTP, and GTP and either limiting ([UTP] = 10 µM) or saturating ([UTP] = 1 mM) amounts of UTP for various times (Fig. 3). An analysis of pause sites at saturating and limiting [UTP] demonstrates sites that are natural pauses on the template and those that are caused by reduced UTP levels, presumably at sites that contain stretches of Ts in the non-template strand of pfeU1. For example, at both UTP concentrations and all of the time points, pausing occurs at 325 and 450 nt, indicating that these are natural pause sites in the vaccinia E1L and F17R genes. Conversely, pausing occurs at 425, 475t, and 600 nt at 10 µM UTP but these pauses do not occur at 1 mM UTP. The 600-nt paused transcript is believed to correspond to the 15/17 poly(T) site in the non-template strand of pfeU1, but it is difficult to match the template sequence precisely to the transcript sizes due to the presence of the poly(A) heads on the vaccinia virus postreplicative transcripts. It is interesting to note that, at limiting amounts of UTP, it takes ~30 min for the polymerase complex to transcribe to the end of the pfeU1 template (Fig. 3, lane 14) and that the amount of read-through does not appear to increase beyond that time (Fig. 3, compares lanes 14, 15, and 16), although at 60- and 90-min chase times, there is some degradation of larger transcripts (Fig. 3, lanes 15, 16, 31, and 32). The presence of both natural and T-rich pause sites on the pfeU1 template makes it an appropriate tool for investigating transcription of authentic vaccinia virus DNA.

To determine the transcription elongation phenotype of an IBT-resistant mutant, we made transcription extracts from cells infected with a mutant vaccinia virus called IBTr90. In vivo, the IBT^90° mutant virus produces plaques of equal size whether grown in the presence or absence of IBT (28). This mutant maps to the gene encoding the second largest subunit of the viral RNA polymerase, A24R. Sequencing the IBT^90° A24R gene revealed that it contains a Y462H mutation. We initiated a pulse reaction on the pG8GU template with WT or IBTr90 cytoplasmic extracts, washed, and chased the complexes with 1
FIG. 3. UTP concentration affects pausing of polymerase complexes: long template. Transcription complexes were formed on the pfeU1 template during a 20-min pulse reaction with WT extract. Complexes were washed three times in low salt (80 mM KOAc) wash buffer and resuspended in transcription buffer. Complexes were then chased in the presence of 1 mM ATP, CTP, and GTP and either 10 μM (lanes 1–16) or 1 mM (lanes 17–32) UTP for the indicated times.
mM ATP, CTP, GTP, and varying amounts of UTP for 5 or 30 min (Fig. 4). Overall, there is a decrease of ~40% in the total amount of transcripts produced in a pulse reaction with the IBTr90 polymerase complex compared with the amount produced by the WT complex (Fig. 4, compare lanes 1 and 18) (data not shown). There is also a decrease in the percentage of full-length transcripts produced by the IBTr90 polymerase compared with those produced by WT as is shown in the graphs of percentages of full-length transcripts versus chase time, which were calculated for two separate experiments (Fig. 4, A and B). For example, in the WT 5-min chase at 30 μM UTP, roughly half of the transcripts are paused and half of them are full length, whereas in the IBTr90 5-min chase at 30 μM UTP, only 18% transcripts are full length (Fig. 4, A, compare lanes 4 and 21, and B). However, the IBTr90 elongation defect only appears at limiting UTP concentrations (Fig. 4A, compare lanes 3–8 with lanes 20–25 and lanes 11–15 with lanes 28–32, B, and C). At 1 mM UTP in a 5-min chase and at 250 μM and 1 mM UTP in a 30-min chase, there is little difference in the amount of full-length transcripts produced by the WT and IBTr90 polymerases (Fig. 4, A, compare lane 9 with lane 17 and lanes 25 and 26 to lanes 33 and 34, B, and C). These results indicate that the IBTr90 polymerase complex is defective in elongation compared with the WT complex.

To determine whether IBTr90 was also defective in transcript release, we examined the ability of the IBTr90 mutant polymerase to release full-length transcripts by adding purified recombinant A18 protein, which functions as a postreplicative transcript release factor to WT and IBTr90 reactions (Fig. 5) (22). Complexes were formed on the pG8GU template with WT or IBTr90 extracts, washed, and chased with 1 mM ATP, CTP, GTP, and UTP for 30 min with increasing amounts of purified A18 protein added to the chase reaction (Fig. 5A). For both the WT and IBTr90 reactions, an increase in the amount of A18 protein added to the chase reaction results in an increase in the amount of released transcripts (Fig. 5A, B, and C). There was no difference in the percentage of released transcripts at any given A18 concentration between the WT and IBTr90 reactions (Fig. 5B). Therefore, the IBTr90 mutation only affects transcription elongation and not transcript release. There are several possible explanations for this transcription elongation defect including an inability of IBTr90 to cleave the 3′-O-methyl-GTP residue incorporated into the nascent transcript at the end of the 37-nt G-less cassette in this template, an inability to recover from a pause, a decrease in

**Fig. 4.** IBTr90 is defective in transcription elongation. A, transcription complexes were formed on the pG8GU template during a 20-min pulse reaction with either WT or IBTr90 extract (Pulse). Complexes were washed three times in low salt (80 mM KOAc) wash buffer and resuspended in transcription buffer. Complexes were then chased in the presence of 1 mM ATP, CTP, and GTP and varying amounts of UTP, as indicated, for 5 (lanes 2–17) or 20 min (lanes 19–34). Transcripts from complexes stalled at the 9-nt poly(T) site are labeled Paused, whereas transcripts from complexes that reached the end of the template are labeled Full-length. B, graph of the percent full-length transcripts after a 5-min chase for the WT (black) and IBTr90 (hatched) reactions. C, graph of the percent of full-length transcripts after a 20-min chase for the WT (black) or IBTr90 (hatched) reactions. Error bars indicate the results of two independent experiments.
stability of the polymerase at the pause site, or a decrease in the affinity of the polymerase for NTPs.

The addition of IBT to a WT vaccinia virus infection produces postreplicative transcripts that are longer than normal, indicating that IBT has a positive effect on elongation in vivo. We tested the effect of IBT in the in vitro transcription assay using the pG8GU template by preincubating WT or IBTr90 cell extracts with IBT with at 30 °C for 30 min or by adding IBT to the chase (data not shown). No effect of IBT was noted in either case for the WT or IBTr90 reactions. In addition, cytoplasmic extracts were made from cells infected with the WT or IBTr90 virus in the presence and absence of IBT and used in the in vitro transcription assay on the pG8GU template (data not shown). Again, the presence or absence of IBT in cytoplasmic extracts had no effect on in vitro elongation of WT or IBTr90 polymerase complexes. The lack of IBT activity in vitro could indicate that IBT needs to be modified in some way to be active and that the design of the in vitro experiments that were conducted did not allow for this modification. It is also possible that IBT targets a protein that was not present in the in vitro elongation complexes.

To further investigate the elongation defect of the IBTr90 mutant, we performed a fine time course experiment to examine read-through from the pulse reaction through the pause site to the end of the template (Fig. 6). Transcription pulse reactions were initiated with either WT or IBTr90 cell extracts on the pG8GU template, washed, and chased with either 30 μM or 1 mM UTP for the indicated times (Fig. 6A). The percentage of full-length transcripts versus time was graphed (Fig. 6, B and C). In this figure, the film exposure times of the WT reactions and the IBTr90 reactions were adjusted to compensate for the reduction in overall transcription by the IBTr90 polymerase and to allow easier visualization of the elongation defect of IBTr90. As in the previous experiment, there are lower percentages of full-length transcripts versus time for the WT reactions and the IBTr90 reactions than for the IBTr90 reactions (Fig. 6, A, compare lanes 2–9 with lanes 17–24 and lanes 10–15 with lanes 25–30, B, and C). In both the WT and IBTr90 reactions at 30 μM and 1 mM, transcripts that are arrested at the end of the 37-nt G-less cassette are present at 30 s and 1 min into the chase and there is no difference in the amount of time it takes for either polymerase complex to elongate from a pulsed to a paused complex (Fig. 6, A, compare lanes 1 and 2 with lanes 17 and 18 and lanes 10–15 with lanes 25–26, and D). These results indicate that the mutation in IBTr90 does not affect that the ability of polymerase to cleave the 3’-O-methyl-GTP that is incorporated at the end of the G-less cassette but does affect its ability to resume elongation from a paused state.
We next compared elongation of WT versus IBTr90 polymerase complexes on the pfeU1 template (Fig. 7). Pulse reactions were initiated on pfeU1 with WT or IBTr90 cell extracts, washed, and chased for 2 or 10 min with varying amounts of UTP. To obtain equivalent exposures for the WT and IBTr90 reactions, we loaded only one-half of the WT reactions on the gel. The IBTr90 elongation defect detected on the pG8GU template is also evident on the pfeU1 template. At limiting concentrations of UTP, the WT polymerase produces longer transcripts than the IBTr90 polymerase. For example, at 30 μM UTP in a 2-min chase, the WT transcripts reach 600 nt in length, whereas the IBTr90 transcripts reach only 350 nucleotides (Fig. 7, compare lanes 4 and 16). However, as demonstrated with the pG8GU template, there is little difference in the length of transcripts produced by WT or IBTr90 polymerase complexes at saturating levels of UTP (Fig. 7, compare lane 6 with lane 18 and lane 12 with lane 24). Paused complexes are present in all of the reactions but differ slightly in the IBTr90 reactions. Most notably, there is an accumulation of paused IBTr90 transcripts at ~425 nt at low UTP concentrations (Fig. 7, lanes 20–23). Presumably, this site is a poly(T) site in the non-template strand of pfeU1 because the amount of paused transcripts decreases as levels of UTP increase.

Destabilizing agents such as salt, sarkosyl, and heparin can be used to assess the stability of a polymerase complex. In early transcription, initiation complexes are sensitive to very low levels of sarkosyl, whereas elongation complexes are more resistant to sarkosyl and salt (39). If the IBTr90 elongation defect were due to a decrease in the stability of the IBTr90 polymerase complex on the DNA template, we would expect to see a lower percentage of full-length transcripts and a higher percentage of transcripts released at the pause site in the IBTr90 reactions in response to high concentrations of salt, sarkosyl, or heparin in the chase reaction. To test this possibility, we initiated pulse reactions with WT or IBTr90 extracts on the pG8GU template, washed the transcription complexes, and chased with 30 μM UTP and varying concentrations of NaCl (Fig. 8). We separated the bead-bound transcripts (B) from the released transcripts (R) after the chase reaction and used the ratio of bound to released transcripts as a measure of polymerase stability. The elongation defect of the IBTr90 polymerase is evident in the absence of NaCl, because only 27% IBTr90 transcripts are full length, whereas 55% WT transcripts are full length (Fig. 8, A, lanes 2 and 15, and B). We noted three general effects of NaCl on both WT and IBTr90 elongation complexes. The first effect is that NaCl decreases the percentage of full-length transcripts even at the lowest salt concentration (Fig. 8B). There continues to be an increase in the amount of paused transcripts as the salt concentration is increased from 100 to 400 mM NaCl, at which point >95% of both WT and IBTr90 transcripts are
paused. The second general effect of NaCl in this assay is to decrease the percentage of bound transcripts. The most substantial effect occurs at NaCl concentrations greater than 300 mM for both WT and IBTr90 transcripts (Fig. 8, A, lanes 4, 5, 17, and 18, B, and C). Finally, transcription elongation to the pause site is fairly resistant to salt, indicating that the elongating polymerase complexes are more stable than the paused complexes (Fig. 8A, lanes 4–9 and lanes 17–22). Overall, the effect of NaCl on the IBTr90 transcription complexes is similar to the effect on WT transcription complexes, indicating that the
stability of these complexes is similar. In addition, the stability of the postreplicative WT polymerase complex in the presence of salt is similar to the stability of the early polymerase complex under similar conditions (39).

As another test of polymerase complex stability, we examined the effect of sarkosyl, an anionic detergent, on elongation. Transcription complexes were assembled on the pG8GU template with WT or IBTr90 cell extracts, washed, and chased with 1 mM ATP, CTP, and GTP, 30 µM UTP, and varying amounts of NaCl, as indicated, for 20 min. Bead-bound transcripts (B) were isolated on a magnet and separated from released transcripts (R). Transcripts from complexes stalled at the 9-nt poly(T) site are labeled Paused, whereas transcripts from complexes that reached the end of the template are labeled Full-length. B, graph of the percent full-length transcripts versus NaCl concentration for the WT (black) and IBTr90 (hatched) reactions. C, graph of the percent bound transcripts versus NaCl concentration for the WT (black) and IBTr90 (hatched) reactions.

In addition to the salt and sarkosyl experiments, we examined the effect of heparin on postreplicative transcription complexes. During early transcription, a WT polymerase complex is...
not able to stably associate with the template DNA if low levels of heparin (\(>0.05\ \mu\text{g/ml}\)) are present prior to the addition of the RNA polymerase (40). In contrast, the vaccinia early elongation protein-DNA complex is extremely stable in the presence of 50 \(\mu\text{g/ml}\) heparin. The WT postreplicative transcription complex was sensitive to the addition of low levels of heparin (10 \(\mu\text{g/ml}\)) in initiation and very stable to high concentrations of heparin (1 mg/ml) in the chase reaction (data not shown). The IBTr\(^{90}\) transcription complex was not tested with regard to heparin stability in initiation but was also stable to high concentrations of heparin during postreplicative elongation.

The elongation rate of the WT vaccinia virus RNA polymerase has been measured from viral cores at 37 °C as a maximum of 17 nt/s (41). To compare the elongation rate of the IBTr\(^{90}\) polymerase with the WT polymerase, we initiated pulse reactions with WT or IBTr\(^{90}\) cell extracts and the pfeU1 template, washed the transcription complexes, and chased with 1 mM ATP, CTP, GTP, and UTP, taking time points every 10 s up to 140 s (Fig. 10). We determined an elongation rate for the WT polymerase of 16.5 nt/s, in good agreement with the previous results. The IBTr\(^{90}\) polymerase has an elongation rate of 8.5 nt/s, which is approximately half of the WT rate. Interestingly, there were differences in the pattern of pausing for WT and IBTr\(^{90}\) polymerase complexes on the pfeU1 template. The chase reaction was done with a saturating concentration of UTP, and therefore, we expect to see only pauses that are not influenced by limiting UTP levels. In general, there are pause sites in the IBTr\(^{90}\) reactions that are not present in the WT reactions, such as those at 225 nt in lanes 17–20, at 250 nt in lanes 17–20, at 525 nt in lanes 21–28, and at just over 600 nt in lanes 21–28 (Fig. 10). There is also enhanced pausing of the IBTr\(^{90}\) polymerase at 310 and 340 nt compared with the WT polymerase. We believe that this indicates that the IBTr\(^{90}\) elongation defect is due to a reduced ability of that polymerase to resume elongation.
tion from a paused state. If the defect were instead attributed to a reduced affinity of the polymerase for NTPs, we would expect to see the reduction in elongation rate for IBTr90 that we observe but not an increase in pausing.

Homology-based computer models can be used to visualize proteins of unknown three-dimensional structure and to assess the structural effect of introducing a mutation into a protein. Because no three-dimensional structure has been determined for the vaccinia virus RNA polymerase, we constructed a homology model for RPO132 based on the closest homolog with a

**FIG. 10.** The IBTr^{90} elongation rate is slower than the WT rate. Transcription complexes were formed on the p6eU1 template during a 20-min pulse reaction with WT (lanes 1–14) or IBTr^{90} (lanes 15–28) extract (Pulse). Complexes were washed three times in low salt (80 mM KOAc) wash buffer and resuspended in transcription buffer. Complexes were then chased in the presence of 1 mM ATP, CTP, GTP, and UTP for the indicated times.
solved structure, the *S. cerevisiae* rpb2 protein (Fig. 11). The ClustalW alignment used for model building indicates that RPO132 and rpb2 share 59.6% amino acid similarity and 21.2% amino acid identity, a degree of relatedness that is near the threshold for model building. Nevertheless, a model was successfully constructed. This model indicates the Y462H mutation present in IBT*90* is located at the base of fork loop 2, a disordered loop that is thought to maintain the integrity of the downstream edge of the transcription bubble (42).

**DISCUSSION**

In this study, we utilized an *in vitro* elongation system to investigate the mechanism of IBT resistance in a vaccinia virus RNA polymerase mutant, IBT*90*. We found that an IBT-resistant virus with a mutation in the A24R gene, which encodes the RNA polymerase subunit RPO132, is defective in postreplicative transcription elongation in *vitro*. Specifically, this mutation causes a reduced ability of the polymerase to resume elongation from a paused state, decreasing the elongation rate of the polymerase by 2-fold compared with WT and increasing the amount of pausing on both natural and artificial DNA templates. The elongation defect does not appear to be a result of a defect in the ability of the mutant polymerase to cleave the 3′ end of an arrested transcript in order to resume elongation, because this cleavage activity was necessary in these experiments to allow the polymerase complex to elongate beyond the end of a 37-nt G-less cassette in the non-template strand of the DNA. In addition, we measured the relative stabilities of the WT and mutant polymerase complexes in response to high salt, sarkosyl, and heparin concentrations. Both enzymes show similar responses to these destabilizing agents, indicating that the IBT*90* elongation defect is not due to a decreased stability of the mutant polymerase complex on DNA.

RPO132 is the second largest subunit of the vaccinia RNA polymerase and has homology to the second largest subunits of both prokaryotic and eukaryotic RNA polymerases. In general, drug-resistant RNA polymerases may result from mutations that directly affect the RNA polymerase by abrogating binding of the drug to the polymerase, as is the case with bacterial RNA polymerases and rifampicin. Mutations may also indirectly affect the RNA polymerase complex by regulating other factors that are utilized in transcription, as is the case with yeast RNA polymerase mutants that are resistant to 6-azauracil, which disrupts the *de novo* synthesis of UTP and GTP (43). In either case, drug resistance may serve as a screening mechanism for mutations that affect transcription.

Several mutations in the β-subunit of the *Escherichia coli* RNA polymerase have been identified that cause increased transcriptional arrest phenotypes and resistance to the antibiotic rifampicin. Rifampicin is a broad-spectrum antibiotic that inhibits bacterial polymerases by binding to the β-subunit of the polymerase and sterically blocking the 5′ end of a nascent transcript that is 2–3-nucleotides long (44). The rifampicin binding pocket is a region that is well conserved in prokaryotes but not in eukaryotes or in vaccinia virus. One *E. coli* rifampicin-resistant mutant, RpoB8, which contains a substitution at an amino acid that interacts directly with bound rifampicin in the β-subunit of the enzyme, was studied extensively *in vitro* (45). The phenotype of the rifampicin-resistant mutant polymerase complex was similar to that of the IBT*90* complex. Specifically, the rifampicin-resistant complex was slower to elongate than a WT complex, paused longer, and paused more frequently than did the WT complex. The authors determined that this mutation specifically increased the *Km* of the polymerase for ATP and GTP, potentially by altering the active site conformation to decrease binding of purines. Although the general phenotype of the RpoB8 mutant was similar to that of the IBT*90* mutant, the RpoB8 mutant reacted differently to an increase of NTPs in the assay. An increase in the NTP levels from 200 to 800 μM allowed the rate of elongation of the RpoB8 polymerase complex to reach that of the WT complex and returned the length and frequency of pauses to a WT level. The overall elongation rate of the IBT*90* complex did not resemble the WT rate even at saturating NTP levels, and IBT*90* continued to pause longer and at more sites than did the WT complex (Fig. 10). These differences support the idea that the IBT*90* mutation specifically affects the mechanism of pause recovery of the vaccinia virus polymerase complex and not affinity of the polymerase for NTPs.

In *S. cerevisiae*, mutations that cause increased transcriptional arrest have been identified in the second largest subunit of RNA pol III, RET-1, and in the largest and second largest subunits of RNA pol II, rpb1 and rpb2. Unfortunately, no crystallographic information is available for the pol III subunit, but a crystal structure is available for the yeast RNA pol II subunit rpb2. Amino acid sequence alignment of vaccinia RPO132 with *S. cerevisiae* rpb2 using ClustalW and building of a homology model indicates that the IBT*90* mutation may map to a region of the rpb2 gene known as fork loop 2 (31, 32). This region is thought to be responsible for maintaining the open complex of DNA during transcription by sterically hindering the reannealing of the DNA at the downstream edge of the transcription bubble. Crystallographic evidence supports this hypothesis, although the exact position of the loop is not clear as six of the residues that comprise it were disordered in the crystal structure (42). The
tyrosine to histidine substitution in IBT may well impinge on the ability of fork loop 2 to maintain the integrity of the transcription bubble. A number of potential mechanisms could explain the loss of function. Substitution of a six-membered ring for a five-membered ring may itself increase the likelihood of transcription bubble reannealing. Alternatively, the RPO132 homology model predicts an alteration of the hydrogen-bonding pattern in the region that may have relatively far-reaching effects on the conformation of RPO132 or its interaction with other subunits (Fig. 11). Finally, the mutation may shift the position of the entire loop or constrain its range of motion. In any case, residues in RPO132 that ordinarily interact with one or both strands of DNA could be moved out of position. This could allow the DNA more freedom to reanneal and, as a result, drive the polymerase toward a backtracked conformation. Regardless of the precise nature of the IBT defect, the function of this region in transcription elongation correlates well with the in vitro transcription phenotype of the IBT mutant and that of the mutants of other species that map to this region as a mutation that affects the ability of the polymerase to maintain the open complex might well be expected to produce an increased pausing/arrest phenotype.

IBT is an anti-poxvirus drug that has been shown in vivo to cause a increase in the size of postreplicative transcripts produced by viruses that are sensitive to the drug. Previous investigations of IBT-resistant vaccinia viruses have not elucidated any effect of IBT on transcript length in vivo (23). However, we propose that in vivo IBT may affect the lengths of transcripts produced by IBT-resistant viruses so subtly as to be difficult to detect. One possible mechanism is that IBT-resistant viruses grown in the presence of IBT produce slightly longer than WT transcripts and that IBT-resistant viruses grown in the presence of IBT produce slightly longer than WT transcripts. In the absence of IBT, the transcripts produced by IBT-resistant mutants would not be so long that they could not encode large proteins, as is the case with IBT-dependent mutants. In the presence of IBT, the transcripts produced by IBT-resistant mutants would not be so long that a deleterious amount of double-stranded RNA would be formed, as is the case with IBT-sensitive viruses. The data presented here support the idea that IBT-resistant viruses make shorter than WT-length postreplicative transcripts. It seems likely that IBT interacts directly with the polymerase complex as rifampicin does with prokaryotic complexes based on the in vivo effect of IBT on WT and IBT-dependent postreplicative transcripts. However, this has not been demonstrated experimentally and no IBT binding site has been identified on the vaccinia RNA polymerase. Another possibility is that IBT acts in a manner only indirectly related to transcription, as is the case with 6-azauracil in yeast (43). IBT could up-regulate a positive transcription elongation factor or down-regulate a negative transcription elongation factor to induce the production of long transcripts. Further studies are needed to investigate the target of IBT and to determine the specific effect of IBT on IBT-resistant viruses in vivo.

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An Isatin-β-thiosemicarbazone-resistant Vaccinia Virus Containing a Mutation in the Second Largest Subunit of the Viral RNA Polymerase Is Defective in Transcription Elongation

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