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Novobiocin Inhibits Vaccinia Virus Replication by Blocking Virus Assembly

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Novobiocin inhibits the replication of vaccinia virus in cultured BSC40 cells. All classes of viral proteins were synthesized during synchronous infection in the presence of drug. The onset of DNA replication was delayed slightly, yet the extent of DNA replication in the presence of novobiocin was comparable to that of a control infection. A delay in the temporal transition to late viral protein synthesis was in keeping with the effects on DNA replication. Although the precursor forms of the major viral structural proteins were synthesized normally at late times, the proteolytic processing of these polypeptides was inhibited, which suggested an impediment to virus assembly. Electron microscopy revealed that novobiocin blocked virus morphogenesis at an early stage. Conversion of the concatameric DNA replication intermediates into hairpin telomeres occurred in the presence of novobiocin, confirming that telomere resolution was not coupled to virus assembly. Novobiocin is the latest addition to a class of antipoxviral agents, which includes rifampin and IMCBH, that arrest morphogenesis.

INTRODUCTION

Vaccinia virus replicates within the cytoplasm of mammalian cells, in near-autonomy of the host cell nucleus. Viral gene expression is regulated in a cascade fashion (reviewed in Moss, 1996). Early genes are transcribed by an RNA synthetic machinery packaged within the infecting virus particle. Disassembly of the virion halts early mRNA synthesis and liberates the DNA genome, which is then replicated by enzymes encoded by early viral genes. Intermediate genes are transcribed after the onset of DNA replication by the vaccinia RNA polymerase plus a new set of accessory transcription factors synthesized during the early stage of infection. Among the products of intermediate genes are factors that reprogram the RNA polymerase to transcribe the late class of viral genes. Each stage of virus macromolecular synthesis is contingent upon proper execution of the preceding phase of viral gene expression.

The assembly of new virus particles commences after the onset of late gene expression. Unlike viral gene expression, which is turned on and off in discrete stages, virus assembly is asynchronous and proceeds continuously during the late phase of the replicative cycle. Formation of the infectious virion and the release of virus particles from the cell have been described at the level of electron microscopy (Morgan, 1976; Dales *et al.,* 1978; Sodeik *et al.,* 1993, 1994). These are events of enormous biochemical and structural complexity that are only just beginning to be understood in molecular terms as methods are developed to arrest morphogenesis at specific steps (for review, see Rodriguez *et al.,* 1997). This has been achieved through the use of drugs that inhibit virus maturation and via the identification of conditional mutants of vaccinia virus that display a morphogenesis phenotype under nonpermissive growth conditions. Viral genes involved in morphogenesis have been identified by various approaches: (i) the isolation of mutant viruses that are resistant to drugs that inhibit assembly and the mapping of drug-resistance loci (Baldick and Moss, 1987; Condit *et al.,* 1991; Meis and Condit, 1991; Schmutz *et al.,* 1991; Tartaglia *et al.,* 1986); (ii) the physical mapping of mutations that confer a temperature-sensitive block to virus assembly (Dyster and Niles, 1991; Ericsson *et al.,* 1995; Kane and Shuman, 1993; Wang and Shuman, 1995; Traktman *et al.,* 1995); and (iii) the engineering of conditional null mutations in genes known to encode structural components of the intracellular mature virus particle (Ravanello and Hruby, 1994; Rodriguez *et al.,* 1995, 1996, 1997; Wolffe *et al.,* 1996; Zhang and Moss, 1991, 1992).

Of the many drugs that inhibit vaccinia virus replication, two compounds — rifampin and *N*-1-isonicotinoyl-*N*-2-3-methyl-4-chlorobenzoylhydrazine (IMCBH) — specifically block virus assembly without grossly affecting viral nucleic acid metabolism or viral protein synthesis. Rifampin causes assembly to arrest after the formation of abnormal membrane-enclosed structures (rifampin bodies), which contain viroplasm, but lack the rigid spherical shape of normal immature particles (Sodeik *et al.,* 1994). The rifampin bodies appear to lack the spicule layer characteristic of the normal immature forms. Rifam-

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pin resistance and hypersensitivity are attributable to point mutations in the D13 gene (Tartaglia *et al.,* 1986; Baldick and Moss, 1987), which encodes a 65-kDa polypeptide. IMCBH inhibits the release of infectious progeny virus particles from the cell by preventing the wrapping of intracellular virions by Golgi membranes (Payne and Kristensson, 1979). IMCBH targets a virus-encoded 37-kDa protein component of the Golgi-derived viral envelope (Schmutz *et al.,* 1991).

In this paper, we describe the inhibitory effects of the coumarin drug novobiocin on the replication of vaccinia virus in cultured BSC40 cells. The coumarin antibiotics, novobiocin and coumermycin A1, have been extensively studied as inhibitors of bacterial DNA gyrase (Maxwell, 1993). Coumarins inhibit DNA gyrase by binding to the gyrB subunit and blocking the interaction of the enzyme with its essential ATP cofactor. Novobiocin and coumermycin are quite potent against gyrase, but also inhibit a variety of other enzymes at higher drug concentrations (Webb and Jacob, 1988; Fox *et al.,* 1996). Antiviral action of coumermycin has been reported against herpes simplex virus type 1 (HSV1) and it was suggested that the viral DNA polymerase might be the drug target (Palu *et al.,* 1986). Novobiocin and coumermycin inhibit HSV1 replication in neural cells and also inhibit virus reactivation from trigeminal ganglia (Spivack *et al.,* 1987).

The inhibitory effects of novobiocin on vaccinia replication *in vivo* (Ikeda *et al.,* 1987) have not been examined in any detail. Vaccinia encodes several potential targets for the coumarin drugs. Of particular interest to us is the vaccinia virus type I DNA topoisomerase, which is inhibited by novobiocin and coumermycin *in vitro* (Fogelsong and Bauer, 1984; Shaffer and Traktman, 1987; Sekiguchi *et al.,* 1996a). We have shown that the coumarin drugs bind to the vaccinia topoisomerase and competitively block the DNA-binding site on the enzyme (Sekiguchi *et al.,* 1996a). The coumarin drugs also inhibit RNA synthesis *in vitro* by vaccinia virus particles (Fogelsong and Bauer, 1984) and by a partially purified transcription complex isolated from virion extracts (Broyles and Moss, 1987). In addition, novobiocin inhibits the vaccinia virus DNA ligase (Shuman, 1996). We now report that novobiocin is indeed an inhibitor of vaccinia replication *in vivo* and that the drug appears to block virus assembly at an early stage. Somewhat surprisingly, we find that novobiocin had relatively little impact on viral gene expression or DNA replication.

MATERIALS AND METHODS

Materials

BSC40 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum. Vaccinia virus WR was routinely propagated in BSC40 monolayers at 37°. Novobiocin, purchased from Sigma, was stored as a 100 m*M* stock solution in water and diluted in DMEM prior to each use.

Viral protein synthesis

Confluent cell monolayers (9.6-cm^2 wells) were infected with vaccinia virus at a multiplicity of 10. The inocula were removed after 30 min and replaced with control medium or medium containing 0.3 or 0.4 m*M* novobiocin. At various times postinfection, the medium was removed, and cells were washed with methionine-free DMEM and then overlaid with fresh medium containing 30 μCi/ml of [^{35}S]methionine (800 Ci/mmol) for 30 min. For cells infected in the presence of the drug, novobiocin was included during the labeling phase. The medium was removed and the cells were lysed *in situ* by the addition of 0.5 ml of a solution containing 0.065 *M* Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, and 10% glycerol. Lysates were stored at −20°. Samples (30-μl aliquots) were heated at 95° for 5 min and then electrophoresed through a 12.5% polyacrylamide gel containing 0.1% SDS. Radiolabeled polypeptides were visualized by autoradiographic exposure of the dried gel.

Processing of viral structural proteins

Synchronously infected cells were pulse-labeled for 30 min with [^{35}S]methionine at 12 hr postinfection as described above. The cells were washed twice with DMEM after the labeling medium was removed and then either lysed immediately (chase time 0) or overlaid with fresh medium containing unlabeled methionine. The monolayers were returned to incubate at 37° and then lysed *in situ* after 1, 2, 4, or 8 hr. Aliquots (40 μl) were analyzed by SDS–PAGE.

Viral DNA replication

Confluent BSC40 cell monolayers (4.0-cm^2 wells) were infected with vaccinia virus at a multiplicity of 10. The inoculum was removed after 30 min and replaced with drug-containing medium. Control infections were performed in the absence of drug. Cells were harvested at 2, 4, 8, 12, and 24 hr postinfection and pelleted in a clinical centrifuge. Cell pellets were resuspended in phosphate-buffered saline and subjected to freezing and thawing, followed by sonication. Aliquots of the lysates were denatured in alkali and applied to a nylon membrane (Zetaprobe; Bio-Rad) using a vacuum slot-blot apparatus. The abundance of viral DNA sequences was assayed by hybridizing to the filter a radiolabeled vaccinia DNA probe synthesized by the nick translation method from a plasmid DNA template containing the vaccinia genomic *Hin*dIII F restriction fragment. Hybridized probe was detected and quantitated by scanning the filter with a FUJIX BAS1000 Bio-imaging analyzer.

Resolution of vaccinia telomeres

Confluent BSC40 cell monolayers (9.6-cm^2 wells) were infected with vaccinia virus at a multiplicity of 10. The inoculum was removed after 30 min. The cells were rinsed twice with DMEM and then overlaid with DMEM (control) or DMEM containing novobiocin. Cells were harvested at 6, 12, and 24 hr postinfection and pelleted in a clinical centrifuge. The cells were resuspended in phosphate-buffered saline and recentrifuged. The washed cells were resuspended in 50 μl of 0.15 *M* NaCl, 20 m*M* Tris–HCl (pH 8.0), 1 m*M* EDTA and then lysed by mixing the suspension with 250 μl of 20 m*M* Tris–HCl (pH 8.0), 1 m*M* EDTA, 0.75% SDS. The lysates were digested with 200 μg of proteinase K for 6 hr at 37°. The samples were extracted sequentially with phenol, phenol:chloroform, and chloroform and then precipitated with ethanol. The nucleic acid was resuspended in 100 μl of 10 m*M* Tris–HCl (pH 8.0), 1 m*M* EDTA and then passed through a 26-gauge needle. The DNA was digested with restriction endonuclease *Bst*EII (50 units, NEB) for 20 hr at 37°. Aliquots of the digests were electrophoresed through a 0.8% agarose gel in TAE buffer (40 m*M* Tris-acetate, 1 m*M* EDTA). DNA was transferred to a Zetaprobe membrane (Bio-Rad) according to the manufacturer's protocol. The membrane was hybridized with a ^{32}P-labeled telomeric DNA probe prepared by nick translation of plasmid pBD6, which contains a *Bst*EII concatamer junction fragment inserted into pUC13 (Merchlinsky *et al.,* 1988). The hybridized probe was detected by autoradiography.

Electron microscopy

BSC40 cell monolayers were infected with vaccinia virus at an m.o.i. of 10. The inoculum was removed after 30 min, and the cells were washed twice with medium and then overlaid with either control medium or medium containing 0.4 m*M* novobiocin. At 24 hr postinfection, cells were dislodged by scraping and then spun in a clinical centrifuge. The supernatant was removed and the cell pellet placed immediately on ice. Cells were fixed initially in 2.5% glutaraldehyde, followed by fixation in 2% osmium tetroxide. Specimens were dehydrated and then embedded in epoxy resin (Polybed 812). Thin sections were stained with uranyl acetate and lead citrate for visualization in a Jeol 1200 CX transmission electron microscope.

RESULTS

Novobiocin inhibits vaccinia replication *in vivo*

BSC40 cells were infected with vaccinia virus at low multiplicity; the inoculum was removed, and the cells were overlaid with medium containing novobiocin in the range 0–0.6 m*M.* Plaque formation was assessed at 48 hr postinfection by staining the monolayers with crystal violet. (Note that addition of drug after removal of the inoculum excluded any possibility that the drug might interfere with virus adsorption.) In control cells without drug, vaccinia formed well-circumscribed clear plaques visible against a background of intact cells (Fig. 1). Inclusion of novobiocin at concentrations $\leqslant$0.1 m*M* had no effect on the number or the size of the plaques (not shown). Plaque size was reduced at 0.2 m*M* novobiocin (Fig. 1). Microscopic plaques were detected at 0.3 m*M* novobiocin. At 0.4 m*M* drug, there was no macroscopic or microscopic evidence of plaque formation (Fig. 1). The cell monolayer was unperturbed by exposure to novobiocin in this concentration range. Toxicity of novobiocin to uninfected cells (manifested as cell rounding and, at higher doses, by dissociation from the plastic surface) was evident at concentrations in excess of 0.6 m*M.*

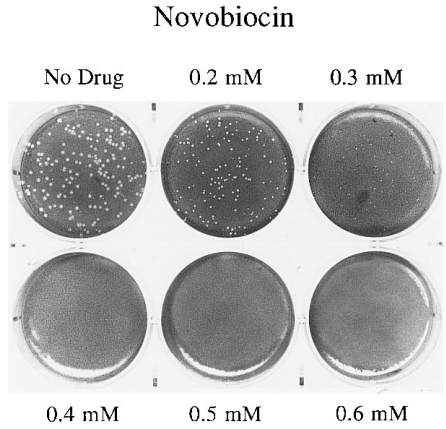

FIG. 1. Novobiocin inhibition of vaccinia virus plaque formation. Confluent BSC40 monolayers were infected with vaccinia WR (~200 PFU/well). The inoculum was removed after 60 min and the cells were then overlaid with medium containing novobiocin at the indicated concentrations. Plaque formation was visualized by staining with 0.1% crystal violet at 48 hr postinfection.

To test if novobiocin inhibition of plaque formation was reversible, cells were exposed to 0.4 m*M* novobiocin for 24 hr postinoculation, at which time the drug-containing medium was withdrawn and replaced with drug-free DME and incubated for an additional 24 hr. Although no plaques had formed after 24 hr in the presence of drug, normal-appearing vaccinia plaques were evident 24 hr after novobiocin was removed (not shown). The number of plaques formed after drug removal was similar to the number seen after 24 hr in a drug-free control. Hence, the antiviral effect of novobiocin was reversible.

Failure to form plaques in the presence of novobiocin may result from inhibition of virus replication or an impediment to virus spread. Thus, we examined the effects of novobiocin under synchronous growth conditions (Fig. 2). A typical one-step growth curve was obtained for control infections performed in the absence of drug; infectious

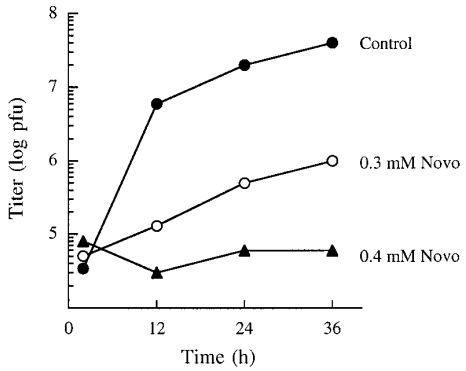

FIG. 2. One-step infection in the presence of novobiocin. Confluent BSC40 cell monolayers (4.0-cm^2 wells) were infected with vaccinia virus at a multiplicity of 10. The inoculum was removed after 30 min and replaced with medium containing 0.3 or 0.4 m*M* novobiocin. Control infections were performed in the absence of drug. Cells were harvested at the indicated times postinfection. Virus yield (log PFU) is plotted as a function of time postinfection.

progeny were detected at 12 hr postinfection and continued to accumulate with time. The yield at 36 hr reflected a burst size of about 50 PFU/cell. Inclusion of 0.3 m*M* novobiocin postinoculation slowed the rate of viral replication and reduced virus yield by a factor of 40 (Fig. 2). At 0.4 m*M* novobiocin, there was no detectable production of infectious progeny above the residua of the inoculum, i.e., the baseline titer at 1 hr postinfection.

Effect of novobiocin on vaccinia DNA replication

Although the results of the experiment depicted in Fig. 2 showed that novobiocin inhibited vaccinia replication, it remained to be determined which intracellular stage(s) of the virus life cycle was affected by the drug. Initial experiments focused on landmark events of viral macromolecular synthesis in synchronously infected cells. The extent of DNA replication was assayed by slot-blot hybridization (Rempel *et al.,* 1990). Cell lysates prepared at various times postinfection in the absence of drug were immobilized on membranes and probed with a radiolabeled vaccinia genomic DNA fragment. Scanning of the hybridized filter revealed low levels of viral DNA at 4 hr, with obvious amplification of viral sequences by 8 hr that continued up to 24 hr (Fig. 3). Inclusion of 0.3 and 0.4 m*M* novobiocin appeared to delay the onset of viral DNA replication by about 1 hr, as judged from the levels of viral DNA at 4 hr and by extrapolation of the curves to the *x*-axis (Fig. 3). Nonetheless, the rates of viral DNA accumulation between 4 and 12 hr were essentially unaffected by 0.3–0.4 m*M* drug (Fig. 3). In this experiment, the yield of viral DNA at 24 hr was reduced by half in the presence of 0.4 m*M* novobiocin. Hence, the complete abrogation of virus production by 0.4 m*M* novobiocin was not the result of drug inhibition of viral DNA replication.

Effect of novobiocin on viral protein synthesis

The patterns of viral protein synthesis in the presence or in the absence of drug were analyzed by pulse-labeling synchronously infected cells with [^{35}S]methionine over a 12-hr time course postinfection. Cell lysates were prepared immediately after the pulse and the profile of newly synthesized polypeptides was examined by SDS–PAGE (Fig. 4). The normal temporal pattern of vaccinia gene expression was seen in the control infections, i.e., the appearance of novel early polypeptides (E) at 2–4 hr postinfection visible against a background of host protein synthesis, transition to the synthesis of distinctive late proteins (L) by 6 hr, and shut-off of host (H) protein synthesis, also by 6–8 hr postinfection (Fig. 4). Exposure to 0.3 m*M* novobiocin had no apparent effect on the timing of viral protein synthesis. At 0.4 m*M* novobiocin, the transition from early to late protein synthesis was delayed slightly, such that characteristic late polypeptides were synthesized prominently at 8 hr rather than at 6 hr (Fig. 4). This is consistent with a slight delay in the onset of DNA replication in the presence of 0.4 m*M* novobiocin; i.e., late protein synthesis requires prior onset of DNA replication. The results presented thus far show that novobiocin had no significant effect on viral gene expression. Hence, the basis for inhibition of viral production must lie elsewhere.

Novobiocin inhibits processing of virion structural proteins

Two major structural proteins of the virion core — 4a and 4b — are encoded by the vaccinia virus A10 and A3 genes, respectively. Each is synthesized at late times as a precursor polypeptide (p4a or p4b) that is proteolytically processed to yield the mature species (VanSlyke *et al.,* 1991a,b; Takahashi *et al.,* 1994). The precursor

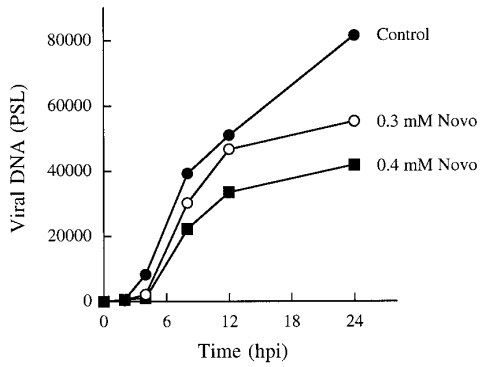

FIG. 3. Effect of novobiocin on viral DNA replication. The amplification of vaccinia DNA as a function of time postinfection was measured by slot-blot hybridization as described under Materials and Methods. The signal intensity of the hybridized probe (PSL, photostimulatable luminescence) was measured by scanning the membrane with a PhosphorImager.

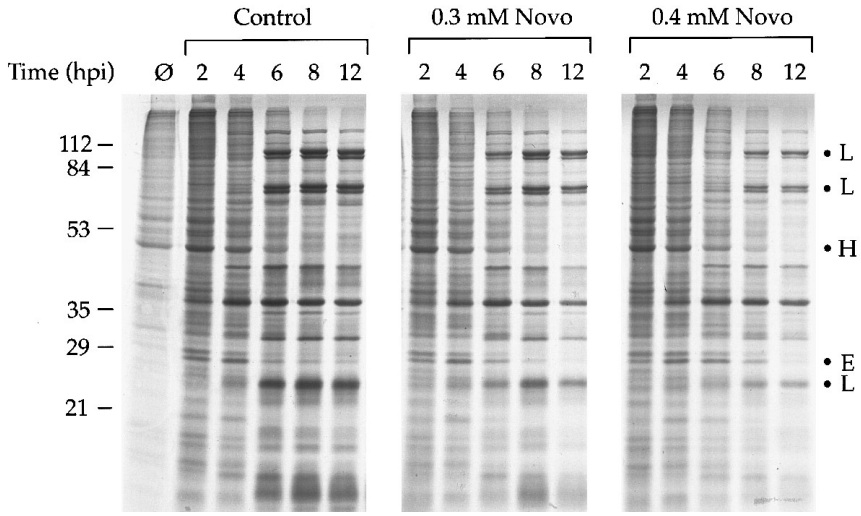

FIG. 4. Effect of novobiocin on viral protein synthesis. Synchronously infected cells were pulse-labeled for 30 min with [^{35}S]methionine as described under Materials and Methods. Monolayers were lysed *in situ* and the labeled polypeptides were analyzed by SDS–PAGE. An autoradiogram of the gel is shown. The infections were performed in the absence of drug or in the presence of 0.3 or 0.4 m*M* novobiocin. The times (hr postinfection) of the pulse labeling are indicated above the lanes. The positions and sizes (in kDa) of prestained molecular weight markers are shown at the left. Representative vaccinia early (E) and late (L) proteins and a prominent host polypeptide (H) are indicated at the right.

polypeptides p4a and p4b were readily identified in [^{35}S]-methionine pulse-labeling reactions performed at 12 hr postinfection and were converted to mature forms 4a and 4b during an 8-hr chase in the presence of unlabeled methionine (Fig. 5, control). p4a and p4b were synthesized during virus infection in the presence of 0.4 m*M* novobiocin, but there was no apparent processing to 4a and 4b, even after 8 hr of chase (Fig. 5, 0.4 m*M* Novo). Because almost any mutation or drug treatment that blocks virus assembly also affects processing of the virion structural precursors, the pulse–chase result provides biochemical evidence that novobiocin affects virus morphogenesis. This was evaluated by electron microscopy.

Electron microscopy

The mature vaccinia virion evolves in stages from microscopically well-characterized immature forms. The earliest of these are crescent-shaped spicule-coated viral membranes, which are observed either free in the cytoplasm or associated with centers of electron-dense

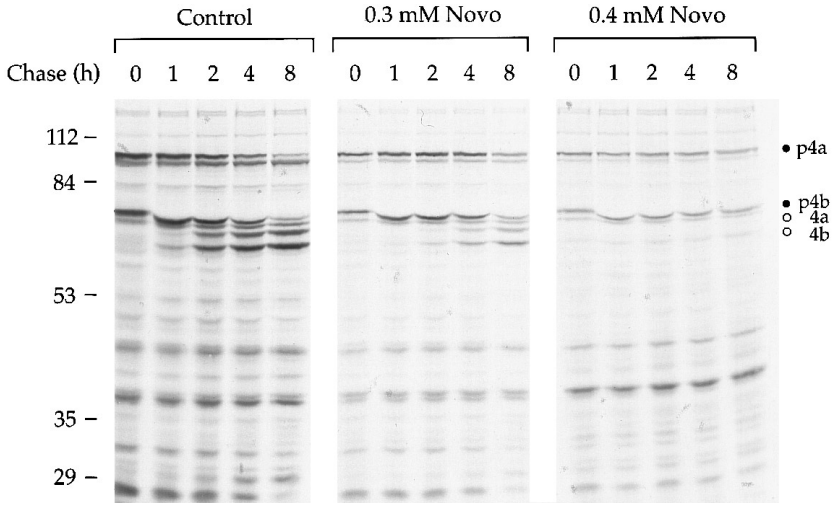

FIG. 5. Defective processing of virion structural proteins in the presence of novobiocin. Virus infections were performed in the absence of drug or in the presence of 0.3 or 0.4 m*M* novobiocin. Cells were pulse-labeled for 30 min with [^{35}S]methionine at 12 hr postinfection and then either lysed *in situ* immediately after labeling (lane 0) or chased for the times indicated (hr) in medium containing unlabeled methionine. Radiolabeled polypeptides were analyzed by SDS–PAGE. An autoradiograph of the gel is shown. The labeled polypeptides corresponding to the structural protein precursors p4a and p4b are indicated by filled circles at right; their processed products 4a and 4b are designated by the open circles.

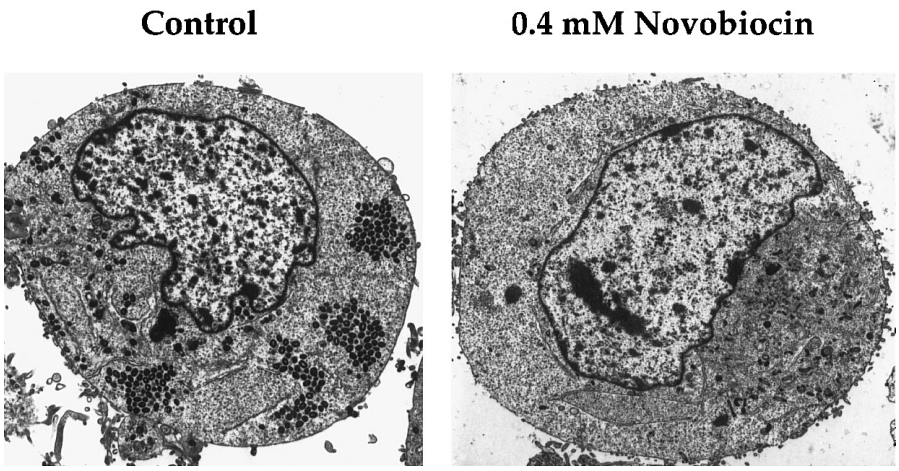

FIG. 6. Novobiocin inhibits the assembly of progeny virions. Virus-infected cells were harvested at 24 hr postinfection and visualized by electron microscopy.

viroplasm. Spherical immature viral particles are formed upon closure of the membrane around the granular material. These spherical immature particles develop into mature brick-shaped virions, clusters of which were evident in the cytoplasm of cells infected with virus in the absence of drug (Fig. 6, Control). Mature progeny virions were rare in cells that were exposed to novobiocin postadsorption (Fig. 6, 0.4 m*M* novobiocin). Spherical immature particles and membrane crescents were rare as well. Thus, the inhibition of vaccinia replication could be attributed to a global defect in virus assembly.

Resolution of vaccinia telomeres

The ends of the 192-kb linear vaccinia genome consist of hairpin telomeres that link the complementary strands into one uninterrupted polynucleotide chain. The telomeres are 104-nt incompletely base-paired structures that exist in two forms ("flip" and "flop") that are inverted and complementary (Baroudy *et al.,* 1982). The telomere structure is formed by resolution of concatemeric DNA replication intermediates into unit-length DNA molecules. The concatemer junctions, which are flanked by large inverted repeats, are converted to mature telomeres by a conservative DNA strand exchange reaction (Merchlinsky and Moss, 1986; DeLange *et al.,* 1986; Merchlinsky, 1990). Although telomere resolution requires the expression of late viral genes (Merchlinsky and Moss, 1989), virtually nothing is known about the identity of specific viral proteins involved in telomere resolution. We analyzed the effects of novobiocin on telomere resolution using the Southern-blotting technique described by Merchlinsky and Moss (1989). DNA was isolated from infected cells at 6, 12, and 24 hr postinfection and then digested with restriction endonuclease *Bst*EII. The digests were electrophoresed through an agarose gel and DNA was transferred to a nylon membrane. The blots were then hybridized with radiolabeled vaccinia DNA containing terminal genomic sequences. *Bst*EII digestion of concatemer replication intermediates yields a 2.6-kb fragment, whereas mature hairpin telomeres yield a 1.3-kb fragment (Fig. 7). An ~11-kb restriction fragment flanking the telomeres also hybridizes to the probe and provides an internal standard for the extent of viral DNA replication (Fig. 7). In control virus-infected cells, most of the telomeric sequences were resolved into the mature form at each time point analyzed. This attests to the high efficiency with which concatemers are resolved *in vivo* and is in agreement with the results of Merchlinsky and Moss (1989).

We again observed a lag in the onset of DNA replication in the presence of 0.3 and 0.4 m*M* novobiocin, reflected in a decreased hybridization signal at the 6 hr time point (Fig. 7). Also, at 6 hr in the presence of novobio-

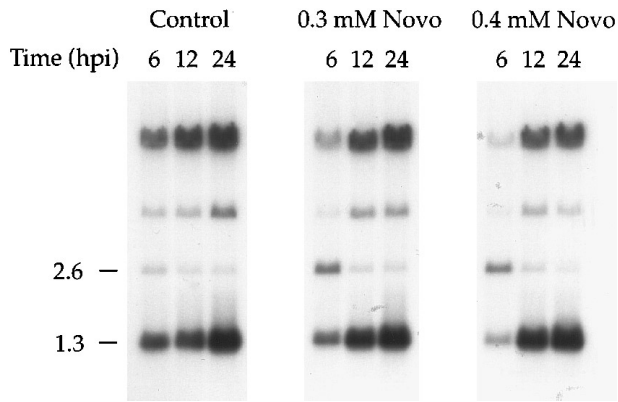

FIG. 7. Telomere resolution. A Southern blot of DNA from virus-infected cells was hybridized with a telomeric probe. An autoradiogram of the blot is shown. The positions of the 2.6-kb concatemer junction fragment and the 1.3-kb mature telomere fragment are indicated on the left.

cin, a substantial fraction of telomeric DNA was in the form of concatemer junctions (Fig. 7). We surmise that this is caused by the delayed onset of late protein synthesis, an event that is essential for telomere resolution. At 12 and 24 hr postinfection in the presence of novobiocin, the extent of DNA amplification was virtually equivalent to that of control cells and the normal high efficiency of telomere resolution was reestablished (Fig. 7). We conclude that novobiocin inhibits morphogenesis, but has no direct effect on telomere resolution. This agrees with the earlier report that rifampin inhibition of virus assembly had no impact on telomere resolution (Merchlinsky and Moss, 1989).

DISCUSSION

The studies presented above indicate that novobiocin inhibition of vaccinia virus replication was caused by a block at an early stage of virus morphogenesis. This effect appeared to be direct, insofar as the drug caused no significant inhibition of viral gene expression or DNA replication. The novobiocin phenotype was similar to those elicited by conditional mutations in viral genes F10 or A17, the products of which are implicated in the initiation steps of viral membrane formation (Wang and Shuman, 1995; Traktman *et al.,* 1995; Rodriguez *et al.,* 1995; Wolffe *et al.,* 1996). Novobiocin appears to act prior to the events targeted by rifampin (acquisition of membrane rigidity) and IMCBH (Golgi wrapping of intracellular mature virus), the two other drugs that specifically block vaccinia assembly.

The assembly defect was unexpected, given earlier findings that the coumarin drugs inhibit a number of vaccinia enzymes involved in DNA and RNA transactions. For example, novobiocin inhibits vaccinia topoisomerase, an enzyme that is essential for virus replication (Shuman *et al.,* 1989). The precise role of the topoisomerase during the vaccinia life cycle remains unclear. We have suggested that topoisomerase functions in telomere maturation by resolving Holliday recombination intermediates (Sekiguchi *et al.,* 1996b). It is obvious from the present study that novobiocin does not affect telomere resolution, at least not when added at 0.3–0.4 mM concentration in the medium. Note that this concentration of novobiocin elicits a 50% inhibition of topoisomerase activity *in vitro* (Sekiguchi *et al.,* 1996a). Hence, topoisomerase may be only marginally affected in our *in vivo* experiments. We doubt that topoisomerase is the *in vivo* target of novobiocin's antipoxvirus activity.

Similar arguments pertain to the relationship of the *in vivo* drug phenotype to the *in vitro* inhibition of vaccinia virus RNA polymerase by 0.5–1 m*M* novobiocin (Fogelsong and Bauer, 1984; Broyles and Moss, 1987). Note that because early mRNAs are synthesized immediately after virus penetration into the cytoplasm, and because our experiments entailed addition of drug 30 min after virus inoculation, we might have overlooked a potential drug effect on viral early gene expression. We addressed this point by pretreating the cell monolayers for 4 hr with 0.4 m*M* novobiocin prior to virus inoculation and maintaining the drug during and after adsorption. We found that pretreatment had no effect on the pattern of early and late viral protein synthesis compared to postinoculation drug treatment. We conclude that 0.4 m*M* novobiocin does not target virus adsorption or virus early gene expression *in vivo*.

How does novobiocin inhibit morphogenesis at an early stage? The membrane crescents are believed to derive from the intermediate compartment — a membrane cisterna situated in the vesicular trafficking pathway between the endoplasmic reticulum and the Golgi stacks (Sodeik *et al.,* 1993). The intermediate compartment hypothesis invokes some molecular "cement" that bridges the two membranes and obliterates the lumen. This could be accomplished either by a single viral protein that transits through both membranes or by two separate proteins that make contact across the lumen. There must be some asymmetry of the fused membrane structure that facilitates the acquisition of curvature, e.g., a specific ligand on the concave face of the crescents which interacts with some other partner. The vaccinia 21-kDa A17 protein, which localizes initially to the rough endoplasmic reticulum and is a component of viral membrane crescents, has emerged as a plausible candidate for initiating membrane reorganization (Rodriguez *et al.,* 1996). A 15-kDa phosphoprotein encoded by the A14 gene is also found in the intermediate compartment and viral membranes (Rodriguez *et al.,* 1997). Conceivably, the F10 protein kinase, which is essential for membrane formation (Wang and Shuman, 1995), may be responsible for phosphorylating A14 and/or other proteins involved in membranogenesis. In this context, any number of specific models can be invoked for novobiocin inhibition of assembly. These include: (i) novobiocin interacts with one of the viral membrane proteins (e.g., A17 or A14) and specifically blocks either its localization or its function; (ii) the drug inhibits the F10 protein kinase; and (iii) novobiocin elicits a nonspecific defect in membrane trafficking. The first two models envision a specific viral gene product as the target of novobiocin action. The classical genetic approach to defining the viral target of drug action would be to isolate a drug-resistant virus mutant (Condit and Niles, 1990). The third model posits a cellular target; this scenario is less amenable to genetics.

It is worth considering the potential utility of coumarin drugs as anti-poxviral therapeutic agents. Although smallpox is no longer extant, the emergence of molluscum contagiosum virus as a common and disfiguring skin infection in AIDS patients illustrates the need for drugs active against the poxviruses (Gottlieb and

Myskowski, 1994). Topical therapy of molluscum contagiosum, i.e., local application of drug to infected skin, eschews systemic toxicity of drugs like the coumarins, which may have pleiotrophic effects on cellular functions at high doses.

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