Adozelesin is a member of a family of extraordinarily cytotoxic DNA damaging agents that bind to the DNA minor groove in a sequence-specific manner and form covalent adducts with adenines. Previous studies employing purified enzymes and adozelesin-modified template DNAs suggested that adozelesin-DNA adducts inhibit DNA replication at the level of nascent DNA chain elongation. In this study, neutral/neutral two-dimensional agarose gel electrophoresis was employed to analyze simian virus 40 (SV40) DNA replication intermediates recovered from adozelesin-treated SV40 virus-infected cells. SV40 replication intermediates rapidly disappeared from infected cells when they were treated with adozelesin, but not when the cells were also treated with aphidicolin to block maturation of replicating SV40 DNA. We conclude that the disappearance of SV40 replication intermediates induced by adozelesin treatment was a consequence of maturation of these intermediates in the absence of new initiation events. Adozelesin inhibition of nascent chain elongation is first observed at concentrations above those needed to block initiation. Adozelesin treatment inhibits SV40 DNA replication at concentrations that produce adducts on just a small fraction of the intracellular population of SV40 DNA molecules.

Adozelesin (U-73,975) is a synthetic analog of the antitumor antibiotic CC-1065 (1). The cytotoxic activity of adozelesin is orders of magnitude more potent than many common antineoplastic agents such as doxorubicin, cisplatin, 5-fluorouracil, or cyclophosphamide. The indole and benzofuran substituents of adozelesin (Fig. 1, subunits B and C, respectively) form non-covalent interactions with DNA that may contribute to the sequence preference of the drug (11, 17–22). These non-covalent interactions cause bending and stiffening of the DNA helix (11–13). After binding to the minor groove, the cyclopropyl ring of the left-hand cyclopropylpyrroloindole substituent (Fig. 1) forms a covalent bond with N-3 of adenine at the 3’ end of the binding site (23, 24). Two consensus sequences have been identified for this alkyla-
tion event: 5’-(A/T)(A/T)A* and 5’-(A/T)(G/C)(A/T)A*, where A* is the alkylated 3’ adenine (9, 22).

Inhibition of DNA synthesis as a result of damage to the DNA template is a common cellular effect of alkylating antitu-
mor agents (25), including adozelesin (3, 5). One possible explanation for this inhibitory effect is that DNA adducts formed by adozelesin and other alkylating agents directly inhibit replication fork progression at the site of adduct formation. This model is supported by cell-free studies, which show that the presence of adozelesin-DNA adducts blocks the progression of DNA polymerase (3, 9, 13) and inhibits helicase-mediated unwinding of the DNA duplex (12, 13).

However, the precise mechanism by which adozelesin inhibits intracellular DNA replication is unknown. In this study, the intracellular effects of adozelesin on DNA replication were examined using simian virus 40 (SV40)-infected African green monkey kidney cells (BSC-1) as a well defined DNA replication model. Except for the virally encoded large tumor antigen, SV40 DNA replication is completely dependent upon the enzymatic machinery of the host cell (26). Furthermore, the examination of DNA damage and drug effects on DNA replication is facilitated by the small (5 kilobase pairs) circular structure of SV40 and the high copy number to which it replicates in infected cells.

The effects of adozelesin on SV40 DNA replication were analyzed by neutral/neutral two-dimensional agarose gel electrophoresis (27), which cleanly separates replicating from non-replicating DNA on the basis of the unique nonlinear structure of DNA replication intermediates (RIs). This technique was used to assay the number and replication status of SV40 RIs isolated from control and adozelesin-treated cells infected with SV40 virus. In addition to examining effects on replication, the number of adozelesin-DNA adducts that form on SV40 DNA at different concentrations of adozelesin was quantitated. The results demonstrated that low concentrations of adozelesin primarily block SV40 DNA replication at the level of initiation of nascent DNA synthesis. Adozelesin treatment inhibits SV40 DNA replication at concentrations that produce adducts on just a small fraction of the intracellular population of SV40 DNA molecules.

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The abbreviations used are: SV40, simian virus 40; BSC-1, African green monkey kidney cells; RIs, replication intermediates; ori, origin of replication; F1, form I DNA; FII, form II DNA; FIII, form III DNA; [3H]Tdr, [methyl-3H]thymidine.
nascnt DNA chains in the absence of significant amounts of adduct formation on SV40 DNA.

EXPERIMENTAL PROCEDURES

Materials—Adozelesin (U-73,975) was obtained from the Upjohn Co. (Kalamazoo, MI). Stock solutions in dimethylacetamide (Aldrich) were dissolved in dimethyl sulfoxide prior to use. Aphidicolin (Sigma) was stored and diluted in 100% ethanol. Stock solutions of both drugs were stored at ~20 °C. Cell culture reagents were purchased from Life Technologies, Inc. unless otherwise specified. Bovine calf serum from defined/supplemented bovine calf serum from HyClone, Proteinase K, restriction endonucleases, and Sephadex G-50 spin columns were obtained from Boehringer Mannheim. High Strength Analytical Grade agarose used for two-dimensional gels was obtained from Bio-Rad. SeaKem LE-agarose (FMC BioProducts, Rockland, ME) was used for forms conversion analyses. DeCAprime II DNA labeling kit was from Ambion (Austin, TX). All other chemicals were of reagent grade or better.

Drug Treatment of Infected Cells and Isolation of SV40DNA—BSC-1 cells were seeded at 5 x 10^5 cells/100-mm plate and grown for 48 h until 70–80% confluent in minimum essential medium with Earle's salts, 0.225% sodium bicarbonate, 1.2 mM L-glutamine, and 10% bovine calf serum. Cells were infected with SV40 virus (multiplicity of infection > 1) diluted 1:10 in minimum essential medium containing 2% bovine calf serum for 2 h at 37 °C. Virus-containing medium was removed, and cells were incubated an additional 22 h in fresh medium. Drugs were added directly to the medium from 100 × concentrated solutions, at the indicated times and concentrations. [3H]TdR (10 μCi/ml medium) was added to each plate for the last 30 min of the drug incubation period. Cells were washed three times with phosphate-buffered saline and incubated for 1 h at 37 °C with 3 ml of 100 μl EDTA and 1% SDS containing 0.2 mg/ml proteinase K. Lysates were scraped into Nalgene Thin-Wall UltraTubes on ice, and 1 ml of 4M NaCl was added to each tube prior to overnight refrigeration. Hirt supernatants were recovered by centrifugation for 30 min at 19,000 x g at 4 °C in an SW-41 ultra-centrifuge rotor and extracted twice with 10 ml of Tris, 1 mM EDTA (TE)-buffered phenol (pH 7.6) and once with chloroform:isoamyl alcohol (24:1). DNA was ethanol-precipitated, recovered by centrifugation as above, washed with 70% ethanol, re-centrifuged, and resuspended in 100 μl of TE. The same DNA samples were used for both replication and forms analyses.

SV40 DNA Replication Analysis—The effects of adozelesin on SV40 DNA RIs were analyzed by neutral/neutral two-dimensional gel electrophoresis using the method of Brewer and Fangman (27) with some modifications. Purified SV40 DNA (10 μl) was linearized with BamHI for 4 h at 37 °C, and electrophoresed in a 0.6% agarose gel in 1 x TAE buffer containing 0.1 μg/ml ethidium bromide (EtBr) for 25 h at 0.7 V/cm. The lanes were excised from the first dimension gel, inserted into enlarged preparative wells in second dimension gels of 1% agarose gel in 0.5 x TBE containing 0.5 μg/ml EtBr, and sealed in place with excess agarose. Second dimension gels were electrophoresed at 4 V/cm in a 1 x TBE running buffer containing 0.5 μg/ml EtBr. Southern blots were hybridized to α-32P-labeled full-length linear SV40 DNA probe (specific activity approximately 1 x 10^6 cpm/μg) prepared by random priming EcoRI-linearized SV40 DNA. Unincorporated dNTPs were removed from labeled probe using a Sephadex G-50 spin column. Blots were hybridized (4 x SSC, 2 x Denhardt's reagents, 0.1% SDS, 0.1% sodium pyrophosphate, 0.011 M sodium EDTA, 0.1 g (w/v) dextran sulfate, and 0.12 mg/ml salmon sperm DNA) to probe for 16 h at 65 °C, washed in 0.2 x SSC, and exposed to a storage phosphor screen (Molecular Dynamics, Sunnyvale, CA). The mass of replication intermediates was determined from two-dimensional gel phosphor images using ImageQuant software (Molecular Dynamics). The fraction of the total population of SV40 molecules that was actively replicating in untreated cells at any given moment was measured by comparing the mass of SV40 RIs with the mass of nonreplicating SV40 DNA isolated from these cells.

For fluorographic analyses, gels were dehydrated by gentle agitation for 1 h in 95% ethanol, followed by a change of ethanol and another 1-h incubation. Gels were impregnated with 5% 2,5-diphenyloxazole in 100% ethanol for 1 h with gentle agitation. Fluor was precipitated during a 45-min incubation in distilled water, and the gels were dried on to Whatman No. 3mm paper using a gel dryer for 60 min at 60 °C. Dried gels were exposed to Kodak XAR-5 film at ~80 °C with exposure times adjusted within the linear response range of the film. Replication activity was determined from the [3H]TDR-labeled DNA signals quantitated from fluorographic patterns scanned by computing laser densitometer (Molecular Dynamics).

For analysis—10 μl of sample DNA was heated for 2 h at 70 °C to inactivate strand damage at the sites of adozelesin adducts. As we have demonstrated with the adozelesin parent drug CC-1065, under these conditions, maximum induction of DNA strand damage was obtained with minimum degradation of control DNA samples (28). Samples were then electrophoresed on a 1% agarose gel in 1 x TAE running buffer (50 mM Tris-HCl, 66 mM acetic acid, and 2 mM EDTA, pH 8.3) at 0.8 V/cm for 16 h at room temperature. Gels were stained with 0.25 mg/ml EtBr and checked for equal loading of DNA per lane using an ultraviolet transilluminator. Gels were either Southern blotted and hybridized to examine drug effects on SV40 DNA forms or fluorographed to evaluate replication activity using the methods described under "SV40 DNA Replication Analysis." Relative amounts of forms (supercoiled (I), relaxed (II), and linear (III)) were quantitated from phosphor images of hybridized blots using a PhosphorImager and ImageQuant software.

[3H]TDR-labeled DNA was quantitated from fluorographs using a computing laser densitometer and ImageQuant software.

RESULTS

Adozelesin Effects on Initiation of SV40 DNA Replication—To directly examine the effect of adozelesin treatment on SV40 DNA replication, SV40-infected BSC-1 cells were treated with various concentrations of adozelesin for 2 h beginning 24 h after infection. A 2-h incubation time was chosen because it had previously been determined that this was the requisite time for the parent compound, CC-1065, to form the maximum number of DNA adducts in infected BSC-1 cells (28). SV40 DNA was pulse-labeled with [3H]TDR for the last 30 min of the drug treatment. SV40 molecules were separated by one-dimensional agarose gel electrophoresis, and incorporation of [3H]TDR into full-length SV40 was measured by fluorography. Fig. 2 shows that replication of SV40 DNA in BSC-1 cells was inhibited by 50% (IC50) at 1 nM adozelesin, and by greater than 2 logs with 10 nM drug.

To determine if the inhibitory effect of adozelesin on intra-cellular SV40 DNA replication occurs at the level of initiation or elongation of nascent DNA chains, we employed a neutral/neutral two-dimensional agarose gel electrophoresis technique for analyzing DNA replication intermediates (27). This method separates branched replicating molecules (RIs) from linear, non-replicating molecules on the basis of size in the first dimension, and size and shape in the second dimension. The nonlinear shape of replicating molecules retards their migration in the second dimension compared to non-replicating linear molecules. Replicating SV40 DNA molecules, which have been cleaved at a single site (BamHI) in the termination region, contain two replication forks positioned at equal distances from the origin of replication where DNA replication initiates (Fig. 3). A mixed population of these molecules replicated to various extents will produce an arc (“bubble arc”) in neutral-neutral two-dimensional gels that rises upward and to one side of the
"In spot," which is where linearized full-length non-replicating molecules of SV40 DNA migrate (Fig. 3). Densitometric quantitation of the signal in the bubble arc and in the in spot indicates that, on average, approximately 12% of the total population of intracellular SV40 DNA molecules are replicating at any given time in these experiments.

Two-dimensional gel analysis was performed on SV40 RIs recovered from SV40 infected BSC-1 cells treated with various concentrations of adozelesin for two hours. Concentrations of drug ranged from doses that had little or no effect on incorporation of \(^{3}H\)TdR into full-length SV40 to those that were nearly completely inhibitory. When probed blots of two-dimensional gels containing replicating SV40 DNA from untreated control cells were exposed to a PhosphorImager screen, SV40 RIs were observed to migrate predominantly as a bubble arc, as expected (Fig. 4). Treatment of infected cells with adozelesin caused a concentration-dependent decrease in SV40 RIs detected in the bubble arc. A marked reduction in SV40 RIs was observed with 2 and 10 nM adozelesin, and SV40 RIs were virtually undetectable after 50 nM adozelesin treatment. No evidence was obtained for replication fork destabilization, which would cause an increase in signals from single fork arcs and broken bubble arcs in neutral-neutral two-dimensional gels (Fig. 3). This contrasts with the replication fork destabilization observed when SV40-infected cells are treated for prolonged periods of time with compounds that block nascent DNA chain elongation (29, 30).

To assess the time frame with which adozelesin caused a decrease in SV40 RIs, this analysis was repeated with SV40 DNA recovered from virus-infected cells that had been treated with 50 nM adozelesin for various lengths of time ranging from 30 min to 2 h. No measurable loss of SV40 RIs was observed after 30 min of treatment (Fig. 5). Treatment of cells with adozelesin for 60 min reduced the numbers of SV40 RIs to less than half the number observed in untreated control cells (Fig. 5). Similar to the data in Fig. 4, SV40 RIs were barely detectable after treatment with 50 nM adozelesin for 120 min.

The rapid disappearance of SV40 RIs in these experiments might have been related to the maturation of SV40 RIs into full-length linear SV40 DNA in the absence of initiation of new RIs. Alternatively, SV40 RIs might have disappeared in adozelesin-treated cells due to their rapid destabilization. Although the absence of an increase in signals from the two-dimensional gel fork and broken bubble arcs associated with replication fork
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Fig. 5. Intracellular effects of adozelesin exposure time on SV40 DNA replication intermediates. SV40-infected BSC-1 cells were treated with 50 nM adozelesin for the indicated times (0, 30, 60, or 120 min). Hirt supernatants were prepared from cell lysates. Replication intermediates were separated by two-dimensional agarose gel electrophoresis and detected by Southern blotting and hybridization to 32P-labeled full-length SV40 DNA. Each pattern is from the same experiment and is representative of three to four independent experiments.

destabilization (31) suggested the first explanation was the correct one, we performed an additional experiment to distinguish between these possibilities. In this experiment, SV40 RIs were isolated from adozelesin-treated SV40-infected cells that were also treated with 5 μM replication inhibitor aphidicolin beginning 5 min prior to the addition of 50 nM adozelesin for 2 h. Aphidicolin inhibits the chain elongation phase of DNA replication by blocking DNA polymerization (32–34). If SV40 RIs disappeared from adozelesin-treated cells because they matured in the absence of new initiation events, aphidicolin’s ability to inhibit elongation should block this maturation, and equal numbers of SV40 RIs should be observed in cells treated with both aphidicolin and adozelesin compared to those treated with aphidicolin alone. In contrast, adozelesin-induced replication fork destabilization should cause SV40 RIs to disappear even when replication forks are first arrested by aphidicolin.

As before, treatment with 50 nM adozelesin alone caused the complete disappearance of RIs from SV40-infected cells (Fig. 6). However, substantial numbers of SV40 RIs were recovered from infected cells treated with both aphidicolin and adozelesin, and with aphidicolin alone (Fig. 6). This result demonstrated that the disappearance of most of the SV40 RIs in adozelesin-treated cells was, in fact, due to their maturation into fully replicated DNA. The small decrease in numbers of SV40 RIs recovered from infected cells treated with aphidicolin compared to control cells may reflect the partial destabilization of replication forks observed previously in association with replication arrest induced by more prolonged treatment with aphidicolin (29, 30). However, the recovery of similar numbers of intact SV40 RIs from aphidicolin/adozelesin-treated cells compared to those treated with aphidicolin alone demonstrated that adozelesin did not cause further destabilization of replication forks.

Adozelesin Effects on Inhibition of SV40 Replication Fork Progression—While adozelesin’s initiation inhibitory effect was not accompanied by a substantial inhibitory effect on the elongation of nascent SV40 DNA chains, it was possible that our experiments did not detect a partial inhibitory effect on elongation which caused replication forks to slow, rather than completely stall. This possibility was tested by measuring the replication activity of residual RIs present at concentrations and times of exposure to adozelesin that are only partially inhibitory to SV40 DNA replication. Replication activity was measured by fluorography of two-dimensional gels containing BamHI-digested SV40 DNA pulse-labeled with [3H]TdR and purified from control and adozelesin-treated cells. The fluorograph analyses were performed with aliquots of the same samples that were used to determine the number of RIs by Southern blotting of two-dimensional gels (Figs. 4 and 5). Measurements were made of incorporation into RIs rather than fully replicated molecules in order to account for small decreases in the number of RIs that sometimes occur during their isolation due to the inherently unstable nature of replicating DNA structures (35). This analysis provided a measure of the relative specific activity of pulse-labeled SV40 RIs. Relative specific activity is defined as the amount of [3H]TdR incorporated per SV40 RI recovered from adozelesin-treated cells compared to incorporation per SV40 RI in untreated control cells. A partial elongation inhibitory effect should produce replication forks that have a lower relative specific activity compared to controls, (i.e. incorporate less [3H]TdR per SV40 RI compared to unimpeded replication forks pulse-labeled in untreated cells). In contrast, the absence of an effect on elongation would produce a population of SV40 RIs, which, while reduced in numbers, contains the same amount of [3H]TdR per RI as control cells.

Visual inspection of the fluorographs in Figs. 7 and 8 showed that the decreased incorporation of [3H]TdR into SV40 RIs observed at various concentrations and times of adozelesin treatment was generally similar to the decreases in the amount of SV40 RIs observed in the Southern blots from two-dimensional gels containing the same samples of DNA (Figs. 4 and 5, respectively). In fact, densitometric quantitation of the PhosphorImager bubble arc signals obtained from hybridized blots (Figs. 4 and 5) and of the fluorograph signals obtained from multiple exposures of fluorographs to x-ray film (Figs. 7 and 8) revealed that decreases in incorporation at 0.5 and 2.0 nM adozelesin were identical to decreases in the numbers of RIs
observed at these concentrations of drug (Fig. 9, panel A). Similarly, decreases in incorporation observed after 30 and 45 min of treatment with 50 nM adozelesin also were identical to the decreases in the numbers of RIs observed at these time points (Fig. 9, panel C). Thus, the relative specific activity of these RIs remained unchanged, indicating that the inhibition of SV40 initiation observed at these drug concentrations and time points is not accompanied by an inhibitory effect on elongation of nascent chains. This result contrasted with the result obtained by pulse-labeling infected cells treated with the elongation inhibitor aphidicolin. In this case, the relative specific activity of SV40 RIs was observed at higher concentrations (Fig. 9, panel A) and at later time points (Fig. 9, panel C), suggesting that further accumulation of drug adducts can lead to an inhibitory effect on elongation. However, this inhibitory effect was observed only after 70–90% of the SV40 RIs present in untreated cells had matured in the absence of new initiation events in cells treated with high concentrations of adozelesin (Fig. 9, panels A and C). Therefore, the predominant inhibitory effect of adozelesin treatment on SV40 DNA replication occurs at the level of initiation of new DNA chains.

Evaluation of Adozelesin-induced Damage to SV40 DNA—Since cell-free studies showed that adozelesin-DNA adducts inhibit DNA polymerase progression (3, 9, 13), and helicase-mediated unwinding of the DNA duplex (11, 13), it was expected that adozelesin treatment would cause an inhibitory effect on elongation of nascent chains. One possible explanation for the lack of an elongation inhibitory effect at lower drug concentrations and earlier time points was that the number of adozelesin adducts formed on SV40 DNA molecules was not sufficient to block replication fork progression. To address this possibility, forms conversion analysis was employed to determine the extent of intracellular SV40 DNA alkylation by adozelesin. Heating DNA containing adozelesin adducts produces
The indicated concentration of adozelesin (0, 0.5, 2, 10, 50, or 200 nM). Hirt supernatants were prepared from cells lysates, and thermally labile adozelesin strand damage was induced by heating for 2 h at 70 °C. The resultant SV40 forms were isolated by agarose gel electrophoresis. A, representative Southern blot hybridized to 32P-labeled full-length SV40 DNA; B, quantitation of forms conversion from hybridization blots where the concentrations of supercoiled form (F) I (●), nicked circular FIII (●), and linear FIII (▲) are expressed as the percent of the total concentration of DNA forms in each sample. Each data point in the graph represents the mean ± S.E. (n = 3-5).

Fig. 10. Intracellular effects of adozelesin concentration on SV40 DNA forms. SV40-infected BSC-1 cells were treated for 2 h with the indicated concentration of adozelesin (0, 0.5, 2, 10, 50, or 200 nM). Hirt supernatants were prepared from cell lysates, and thermally labile adozelesin strand damage was induced by heating for 2 h at 70 °C. The resultant SV40 forms were isolated by agarose gel electrophoresis. A, representative Southern blot hybridized to 32P-labeled full-length SV40 DNA; B, quantitation of forms conversion from hybridization blots where the concentrations of supercoiled form (F) I (●), nicked circular FIII (●), and linear FIII (▲) are expressed as the percent of the total concentration of DNA forms in each sample. Each data point in the graph represents the mean ± S.E. (n = 3-5).

The nature of the extraordinary cytotoxicity of adozelesin and related alkylating minor groove binding drugs is not known. Like many antitumor agents, these compounds inhibit DNA replication, and one possibility is that their cytotoxicity is related to the inhibitory effect they have on this fundamental cellular process. As part of our continuing effort to better understand the cytotoxic effects of this and related compounds, the objective of this study was to determine the mechanism by which adozelesin inhibits SV40 DNA replication.

Cell-free DNA replication studies have shown that adozelesin-DNA adducts block the progression of bacterial polymerases along template DNA and inhibit the unwinding of duplex DNA by purified helicases (3, 9, 11–13). We recently showed that CC-1065, which is another member of the cyclopropylpyrroloindole class of compounds, forms DNA adducts at specific sites in the SV40 genome in SV40-infected cells (28). With this in mind, our initial two-dimensional gel analysis was designed to detect SV40 DNA replication forks accumulated at specific sites due to the ability of adducts to arrest DNA polymerization or unwinding of template DNA.

Contrary to our expectation that adozelesin treatment would cause SV40 RIs to accumulate, we observed a dose- and time-dependent reduction in SV40 RIs in adozelesin-treated cells (Figs. 4 and 5). This contrasts with the large numbers of SV40 RIs observed by two-dimensional gel analysis when the maturation of SV40 RIs has been blocked by treatment with elongation inhibitors such as aphidicolin (31). In fact, large numbers of SV40 RIs were observed in adozelesin-treated cells when these cells were also treated with aphidicolin to block maturation of previously formed replication intermediates (Fig. 6). These results definitively establish that the reduction in SV40 RIs observed in association with adozelesin treatment alone is caused by the maturation of SV40 RIs in the absence of new initiation events. Furthermore, when tritiated thymidine incorporation into residual RIs was measured in cells treated with partially inhibitory doses of adozelesin, it became apparent that adozelesin's inhibitory effect on initiation of SV40 DNA replication is not accompanied by significant inhibitory effect on nascent DNA chain elongation (Fig. 9).

The nature of the inhibitory effects on initiation of SV40 DNA replication induced by adozelesin is not clear. The drug has no detectable reactivity with protein (9), and DNA adduct formation by the cyclopropyl moiety of adozelesin and other members of this family of drugs is essential for the biological activity of these compounds (1). Therefore, adozelesin's inhibitory effects on initiation are likely related to formation of adozelesin-DNA adducts rather than interactions between adozelesin and DNA replication proteins or noncovalent interactions between adozelesin and DNA.

Since it is likely that adozelesin-DNA adducts form at specific sequences (similar to its parent compound CC-1065), an alternative possibility is that the expression of genes coding for proteins specifically involved in initiation of SV40 DNA replication is blocked by adducts that selectively form within such genes or nearby regulatory sequences.

These genes most likely would be cellular genes rather than the viral gene, which encodes the single viral protein involved in SV40 DNA replication, large T antigen. This is because the number of adducts formed on SV40 DNA is not sufficient to account for the complete inhibition of large T antigen gene.

DISCUSSION

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expression required to abolish SV40 DNA replication. Furthermore, adozelesin also inhibits cellular DNA replication in mammalian cells in the absence of viral proteins. In either case, the fact that significant inhibition of RI occurs within 1 h suggests that the cellular or viral proteins coded for by these genes would have rapid turnover rates.

Alternatively, adozelesin's inhibitory effects on initiation of SV40 DNA replication may result from the induction of a cellular DNA damage response that occurs independently of where adozelesin-DNA adducts are formed. For instance, it was recently demonstrated that inhibition of DNA replication in budding yeast by the DNA damaging agent methyl methanesulfonate is part of a checkpoint response mediated by the RAD53 and MEC1 genes (36). We recently determined that adozelesin also inhibits DNA replication in this organism in a RAD53- and MEC1-dependent manner. Thus, adozelesin's inhibitory effect on DNA replication in budding yeast clearly is related to the S phase checkpoint regulatory response mediated by these two genes.

Whether or not adozelesin's inhibitory effect on initiation of SV40 DNA replication corresponds to a similar response in mammals is not known. However, considerable circumstantial evidence is consistent with this possibility. For instance, SV40 DNA replication also is inhibited in SV40 infected cells or cells containing replicating SV40 episomes when DNA damage is induced in these cells by ultraviolet and ionizing radiation (37). Similarly, DNA damage induced by compounds that methylate DNA inhibits the replication of episomes containing Epstein-Barr virus sequences (38). Similar to the replication arrest induced by adozelesin, DNA damage induced by ionizing radiation (39-41), and DNA methylation (38) inhibit SV40 or Epstein-Barr virus episome DNA replication in association with levels of viral or episomal DNA damage, which are too small to account for replication arrest induced by the formation of adducts on individual molecules of replicating viral or episomal DNA.

Presumably, the inhibition of episomal or viral DNA replication induced by ultraviolet or ionizing radiation is related to the transient decrease in cellular DNA replication, which is also induced by DNA damage (38). In fact, a large number of studies suggest one component of the cellular response to DNA damage induced by these agents corresponds to an inhibitory effect on initiation of new replicons (37). This component is detected at low levels of DNA damage and is also accompanied by an inhibitory effect on elongation when DNA damage is increased.

Similarly, the predominant initiation inhibitory effect on SV40 DNA replication induced by adozelesin at low concentrations is also accompanied by a pronounced inhibitory effect on elongation of nascent DNA chains at higher doses of drug (Fig. 9). Adozelesin also induces a transient arrest of cellular DNA replication in mammals (2, 3), although it is not known whether adozelesin's inhibitory effect on cellular DNA replication occurs at the level of initiation or elongation.

The discovery of a unique mode of action for adozelesin by two-dimensional gel techniques demonstrates the tremendous utility of these powerful techniques for studying at the molecular level, the effects on DNA replication of compounds that target DNA. Most previous attempts to distinguish between initiation- and elongation-specific effects on DNA replication relied on the indirect analysis of nascent DNA intrinsically labeled with radioactive DNA precursor molecules, such as tritiated thymidine. The interpretation of this type of experiment is problematic due to the uncertainties inherent in the analysis of intrinsically labeled DNA. These uncertainties are related to factors such as differences in the rate of fork movement, effects of the size of intracellular precursor pools on radiolabel incorporation, radiolabel incorporation due to DNA repair, and potential inhibitory effects on DNA replication related to the radiosensitive labeling of DNA. In contrast, two-dimensional gel electrophoresis techniques for analyzing DNA replication directly identify replicating DNA independently of an intrinsic label. This feature of two-dimensional techniques is responsible for their enormous effectiveness as tools for studying various aspects of DNA replication in recent years, and made possible detection of adozelesin's initiation-specific effect on SV40 DNA replication.

The application of two-dimensional gel electrophoresis technology to study the effects of other inhibitors of SV40 and cellular DNA replication may identify additional initiation-specific effects similar to the inhibitory effect induced by adozelesin. The ability to selectively inhibit initiation of SV40 DNA replication could prove useful in efforts to dissect the cellular processes involved in initiation of cellular DNA replication, including those steps involved in cellular responses to DNA damage. We are exploring these possibilities, as well as the potential relationship between the initiation-specific inhibitory effect of adozelesin on SV40 DNA replication and the profound cytotoxic effects that may underlie the efficacy of this drug as an antitumor agent.

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