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Cisplatin Resistance and Mechanism in a Viral Test System: SV40 Isolates That Resist Inhibition by the Antitumor Drug Have Lost Regulatory DNA†

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ABSTRACT: Isolates of SV40 that have enhanced ability to survive inhibition by the antitumor drug cisplatin were selected by serial drug challenge in vivo. These mutant viruses have acquired specific deletions within the repeated regulatory motif $(GGGCGG)_6$ or GC box. This DNA element was shown previously to be a strong target of drug attack by cisplatin and other anticancer drugs in vitro and is an important viral and cellular DNA control sequence. Thus, drug resistance in this viral test system is dependent on the loss of important target DNA sequences. The results also indicate that drug efficacy may be related to the ability of certain anticancer drugs to attack regulatory DNA sequences containing strings of guanosines.

Simple platinum compounds such as *cis*-diamminedichloroplatinum(II) (cisplatin) and its derivatives have the ability to selectively inhibit the growth of certain tumor cells [see Nicolini (1988) for a review]. Originally identified as an agent that inhibited *Escherichia coli* cell division (Rosenberg et al., 1965), cisplatin has become one of the most widely used anticancer drugs. The primary functional target of cisplatin attack is believed to be DNA, and the inhibition of DNA synthesis is generally thought to be the process most deleterious to the viability of the tumor cell [reviewed by Sherman and Lippard (1987)]. Recently, however, the central

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role of replication inhibition has been reevaluated, and new mechanisms of action have been proposed (Gralla et al., 1987; Sorenson & Eastman, 1988a).

Most current hypotheses concerning cisplatin action center on its ability to cross-link DNA. Both inter- and intrastrand cross-links have been detected in vitro and in vivo [see Eastman (1987), Sherman and Lippard (1987), and Nicolini (1988)]. These adducts distort the structure of DNA, causing unwinding and compression of the DNA template (Macquet & Butour, 1978; Cohen et al., 1979; Rice et al., 1988). Most attention has focused on the platinum bridge formed between the N-7s of two adjacent guanines on the same DNA strand. This adduct has been demonstrated to inhibit the activity of various DNA copying (Pinto & Lippard, 1985; Gralla et al., 1987) and nucleolytic enzymes in vitro (Tullius & Lippard, 1981; Scovell & Kroos, 1982). Since GG sequences are quite common and dispersed throughout the entire genome, this general mode of inhibition implies that the drug has no preferred chromosomal target regions.

Some studies, however, have suggested that cisplatin may indeed have preferred functional DNA targets (Fraval & Roberts, 1979; Gralla et al., 1987; Sorenson & Eastman, 1988a,b). Although the general inhibition of DNA synthesis remains an attractive model for drug action, a correlation between DNA replication inhibition and cytotoxicity has not always been observed (Salles et al., 1983; Sorenson & Eastman, 1988b). This suggests that the antitumor activity of cisplatin is complex and could affect cell function at more than one level, possibly involving specific gene target sequences.

The most likely specific targets of cisplatin attack are regulatory sequences containing strings of guanosines. In vitro, the drug attacks the GC-rich control elements within the regulatory region of the DNA tumor virus SV40, raising the possibility that related cellular regulatory DNA sequences may be preferred functional targets for cisplatin attack (Gralla et al., 1987). In SV40, these GC-box sequences are within a segment of the regulatory region that is hypersensitive to a variety of DNA modifying reagents in vivo (Beard et al., 1981; Robinson & Hallick, 1982) and the GC-box elements control both viral transcription and replication [see DePamphillis (1988)]. Related cellular sequences control the transcription of cellular genes and are particularly concentrated in the 5′ flanking region of oncogenes [see Dynan and Tjian (1985) and Mattes et al. (1988)]. These same cellular regions are known to become hypersensitive to attacking reagents when the genes or oncogenes are being expressed (Gross & Garrard, 1988). Taken together, these observations allow the possibility that the GC-box or G-string regulatory elements associated with certain critical genes may be preferred targets for anticancer drug attack (Gralla et al., 1987; Mattes et al., 1988).

The clinical use of cisplatin and many other anticancer drugs is hindered by the development of drug resistance in tumors [see Nicolini (1988)]. Many different cellular mechanisms appear to contribute to resistance, including increased adduct repair (Richon et al., 1987; Eastman & Schulte, 1988) and increased levels of glutathione and metallothionen which can potentially inactivate the drug (Teicher et al., 1987; Kelley et al., 1988). In the systems examined to date, the cumulative effects of these and other perturbations are believed to be insufficient to account for the observed levels of cisplatin resistance [see Eastman and Schulte (1988)]. Therefore, it is likely that other unidentified cellular mechanisms exist which can contribute to drug resistance.

The hypothesis that cisplatin attacks and inhibits the function of regulatory DNA elements (Gralla et al., 1987) raises a new possibility, namely, that resistant cells have lost certain critical DNA targets of drug attack. This possibility could eventually be tested in such cells once the range of likely target genes had been identified. In this paper, we apply an alternative initial test by attempting to isolate cisplatin-resistant mutants of simian virus 40. If resistance can be mediated by changing DNA sequences, then the small size of this virus should allow one to characterize the mutations that accompany resistance. The results show that such viruses can be selected by serial drug challenge in vivo and that they have selectively lost viral GC-box DNA.

Experimental Procedures

SV40 Infections in the Presence of Cisplatin. Preconfluent CV-1 cells were infected with 1–5 pfu/cell wild-type 776, SV-P7, or SV-P8 virus for 1–2 h (Buchanan & Gralla, 1987). After the infection period, 10 mL of DMEM, 2% calf serum, and 1% antibiotics (Gibco) were added to infected cells. Cisplatin (Sigma) was dissolved in 0.15 M NaCl (Ciccarelli et al., 1985) immediately prior to use. The drug was stored desiccated (25 °C), and solutions were prepared just before use to expose infected cells to reproducible concentrations of the drug.

A freshly prepared 400 μM cisplatin solution (100–200 μL) was added to the cell medium and allowed to incubate for 8 h in the tissue culture incubator. After the infection period, the infection medium containing cisplatin was removed by aspiration. Infected cell monolayers were washed 2–3 times with warm PBS so that residual extracellular cisplatin and unattached virus were removed. Fresh, cisplatin-free medium was added, and infections were allowed to proceed for 3–4 days. Viral lysates were collected by scraping cell monolayers with a rubber policeman, freeze/thawing 3 times to remove virus particles from cell membranes, and stored at −20 °C.

Development of an SV40 Lysate Resistant to Cisplatin. Infected cells were challenged with 10 μM cisplatin as described above, and the infection was allowed to proceed for 3–4 days. At this time, the viral lysate was harvested and labeled "R1" to designate the first round of infection in the presence of cisplatin. To start the second round of drug selection, new plates of CV-1 cells were infected with the R1 lysate, treated with 10 μM cisplatin, washed, and harvested after 3–4 days. In all, eight such rounds of treatment were done, the first four with 10 μM drug, rounds 5 and 6 with 12.5 μM drug, and rounds 7 and 8 with 15 μM drug. After each round of selection, the number of viable virus particles in each cell lysate was determined by 18-day plaque assay.

Isolation of Plaques from a Cisplatin-Resistant SV40 Lysate, Restriction Enzyme Analysis, and DNA Sequencing of Plaque DNA. The round 8 lysate was subjected to a final ninth round of selection with 20 μM cisplatin using confluent CV-1 cells. At 3–4 days postinfection, an infected cell lysate was prepared and the number of viable virus particles determined by 18-day plaque assay using 0.01% neutral red stain. Seventeen well-isolated viral plaques of nearly uniform size were picked and resuspended in 0.5 mL of PBS and used to infect individual plates of fresh CV-1 cells. To maintain minimal selective pressure, the tissue culture medium was made 5 μM in cisplatin. This concentration of drug inhibits wild-type SV40 growth by approximately 95% (see Figure 2). After an 8-h incubation with the drug, the medium was removed and replaced with cisplatin-free medium, and viral lysates were harvested after 3 days.

Viral DNA was prepared from each of the 17 lysates by infecting confluent plates of CV-1 cells and isolating viral DNA 10 days later. To maximize DNA production, cisplatin

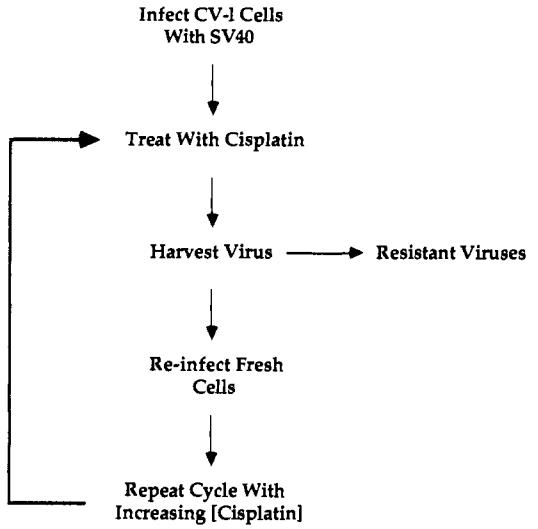

FIGURE 1: Schematic diagram of the protocol used to isolate cisplatin-resistant SV40 viruses.

was not added to infected plates. DNA was isolated from these infected plates, purified, and digested with *Hae*III or *Eco*RII. Nonbanded DNA was sequenced by using a modified dideoxynucleotide chain termination method (Haltiner et al., 1985).

Characterization of Cisplatin-Resistant SV40 Mutant Viruses. The 17 viral isolates were grouped into three sets (SV-P7, SV-P8, SV-P12) on the basis of restriction enzyme analysis and DNA sequencing. Approximately 1–5 pfu/cell SV-P7, SV-P8, or SV-P12 virus was serially diluted and used to infect confluent CV-1 cell plates (6 cm). Infected cells were then exposed to freshly prepared (above) 2–20 μM cisplatin for 8 h. After this time, the drug was removed and replaced with drug-free medium. Infected, drug-exposed monolayers were covered with a 1:1 mixture of 2× DMEM, 2% antibiotics (Gibco), and 1.8% agar (Noble) for plaque formation assay. After 18 days, the agar layer was carefully removed, and monolayers were stained with 0.1% crystal violet to visualize viral plaques. The accumulation of wild-type SV40, SV-P7, and SV-P8 DNA after 8 h of exposure to varying concentrations of cisplatin was estimated by quantitative agarose gel electrophoresis (see legend to Figure 6).

Results

The selection procedure used for the isolation of cisplatin-resistant viruses is illustrated in Figure 1. The monkey kidney cell line CV-1 was infected with SV40 and the infected culture challenged with cisplatin [see Ciccarelli et al. (1985), for example]. After 8 h, the medium was replaced with cisplatin-free medium, and approximately 4 days after infection, infected cell lysates were prepared. These viral lysates were used to infect fresh CV-1 cells, and the challenge and harvest cycle was repeated. Since fresh CV-1 cells were used in each infection cycle, there was no possibility that the cells themselves could acquire resistance and thus mediate increased viral yields. The initial amount of cisplatin used was sufficient to inhibit viral bursts by greater than 95% (see below). The rationale was that mutants that resist inhibition by cisplatin would give greater yields and eventually outgrow the wild-type virus. Yields of viable virus were monitored by 18-day plaque assay after each cycle, and after increases were noted, the concentration of cisplatin in the next cycle was increased. During nine rounds of selection the following concentrations of cisplatin were used: 10 μM for rounds 1–4; 12.5 μM for rounds 5 and 6; 15 μM for rounds 7 and 8; and a final round of selection at 20 μM cisplatin (see Experimental Procedures for further details).

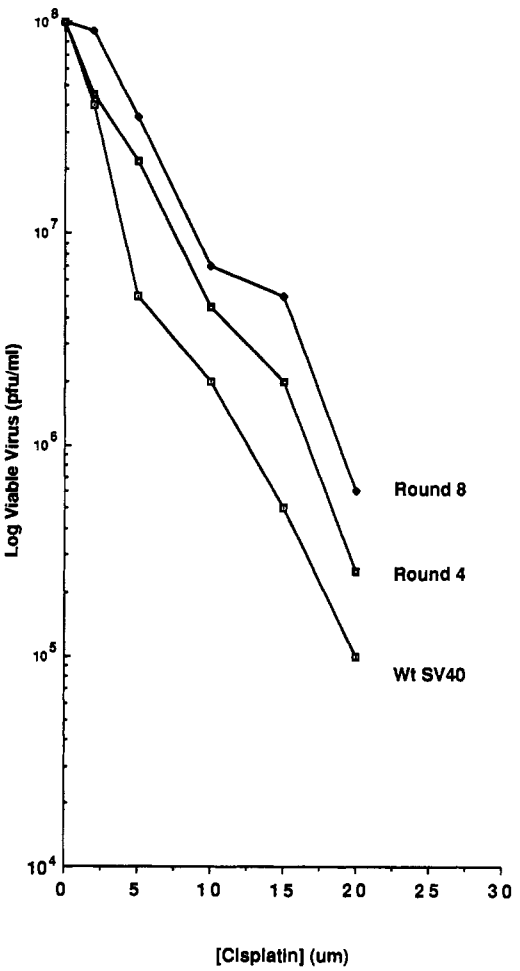

FIGURE 2: SV40 lysates prepared after four and eight rounds of selection with cisplatin show increased survival after cisplatin exposure. Wild-type SV40 and round 4 and round 8 viral lysates were used to infect fresh CV-1 cell monolayers that were subsequently challenged with the indicated amounts of cisplatin as described (see Experimental Procedures). The number of viable virus particles after 3–4 days postinfection was determined by 18-day plaque assay of lysates isolated from the cisplatin-challenged plates.

The viral lysates from rounds 4 and 8 were then assayed systematically for cisplatin resistance. In this experiment a series of infected plates of CV-1 cells were challenged with a range of concentrations of cisplatin. After approximately 3 days, infected cell lysates were prepared and the number of viable virus particles were estimated by 18-day plaque assay. The resulting drug-inhibition curves are shown in Figure 2. The data show that both round 4 and round 8 lysates contain viruses that have an enhanced ability to survive challenge with cisplatin. For example, 99% inhibition of wild-type-virus yield occurs at approximately 13 μM cisplatin, while the R4 lysate requires 17 μM cisplatin and the R8 lysate nearly 20 μM to achieve this same inhibition. We conclude that viruses with enhanced resistance to cisplatin have been enriched by selection and exist in the lysates.

Isolation of Viruses with Enhanced Drug Resistance. The round 9 lysate was used to infect fresh CV-1 cell monolayers in a plaque detection protocol intended to isolate individual virus clones. After 18 days, sufficient time for the appearance of slow-growing viruses, the monolayers were stained with neutral red and individual viral plaques were identified. After this long period of growth, all plaques were of approximately equal size. Sixteen well-isolated plaques were picked, and these

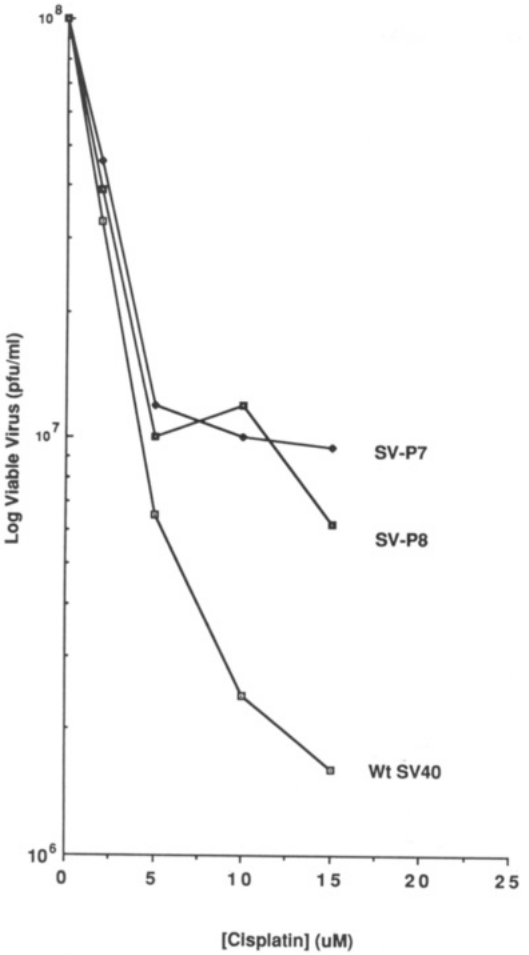

FIGURE 3: Mutant SV40 viruses SV-P7 and SV-P8 resist growth inhibition by cisplatin. 1–5 pfu/mL SV-P7, SV-P8, or wild-type SV40 virus was serially diluted and used to infect CV-1 monolayers. Infected cells were challenged with the indicated concentration of cisplatin for 8 h (see Experimental Procedures). These infected, drug-treated cultures were allowed to incubate for 3–4 days, and then lysates were prepared. The number of viable SV-P7, SV-P8, or wild-type viruses was determined by 18-day plaque assay of the lysates.

were used for isolation of viruses and viral DNA.

DNA from the 16 virus isolates was subjected to preliminary analysis by restriction digestion and by partial sequence analysis of the regulatory DNA (see below). Seven plaques showed changes compared to wild type, and these represented three classes of mutants; SV-P7, SV-P8, and SV-P12 were chosen for further study as representatives of each class. These three viruses were assayed for survivability after a 15 μM cisplatin challenge as an indication of resistance. In this experiment, the number of plaques produced in the presence of cisplatin is compared to the number produced in the absence of drug. The data indicated that viruses SV-P7 and SV-P8 gave an approximately 2.5-fold higher ratio than wild type (an average of five experiments), while SV-P12 showed only a 1.2-fold increase (an average of three experiments). SV-P7 gave higher yields than wild type in the presence of drug in five of five experiments and SV-P8 showed higher yields in four of four experiments. SV-P7 and SV-P8 were thus characterized as viruses that show enhanced ability to resist cisplatin inhibition.

As a further test, a series of plates of CV-1 cells were infected with either SV-P7, SV-P8, or wild-type virus and challenged with increasing concentrations of cisplatin. After 3–4 days, viral lysates were prepared, and the number of surviving viruses was determined by 18-day plaque assay.

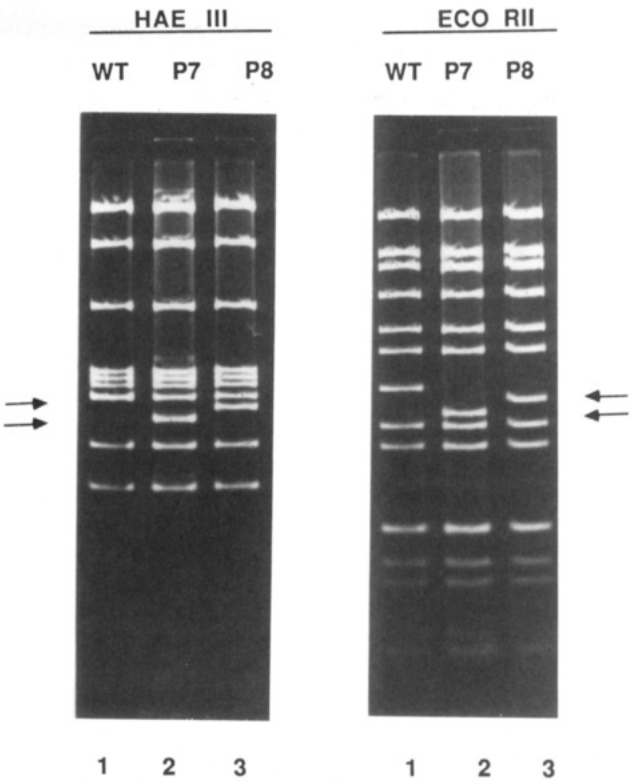

FIGURE 4: *Hae*III and *Eco*RII digestion of wild-type SV40, SV-P7, and SV-P8 DNA. SV-P7 and SV-P8 DNA were digested with either *Hae*III or *Eco*RII and electrophoresed on a 5% polyacrylamide gel. Lane 1 in both panels shows wild-type SV40 digests. Lanes 2 and 3 show SV-P7 and SV-P8 digests, respectively. Arrows indicate the location of the faster migrating origin fragment in SV-P7 and SV-P8 digests.

These numbers were compared to the numbers of viral plaques produced in the absence of drug. The reduction in viable virus yield caused by cisplatin is shown in Figure 3 for the three viruses.

Figure 3 confirms that the mutant viruses resist the effects of cisplatin over the range of concentrations used in the selection process. At low doses of drug both the wild type and the mutants are inhibited similarly. As the level of cisplatin increases, this inhibition continues unabated only for the wild-type virus. By contrast, mutant viruses have acquired the ability to resist the strong inhibition at high drug concentrations. At 10–15 μM cisplatin, the mutants produce 3–5 times the number of viable viruses the wild type produces. Recall that this is the same range of drug dose that was used to select these viruses. We conclude that viruses SV-P7 and SV-P8 were selected because of their ability to better survive cisplatin challenge.

The DNA sequence changes associated with the mutant viruses were then characterized more thoroughly. Figure 4 shows *Hae*III and *Eco*RII restriction digests of SV-P7, SV-P8, and wild type. These enzymes were chosen since their cutting specificity includes GG sequences; recall that the major dinucleotide target of cisplatin attack is this sequence (Sherman & Lippard, 1987; Nicolini, 1988), and the possibility existed that such sequences would be altered in the mutants. The digestion patterns show that all 38 GG sequences assayed (9 GGCC *Hae*III sites and 10 CCA/TGG *Eco*RII sites) are intact in all three viruses. The only indication of an altered DNA sequence is in the faster mobility of one band in each of the mutant DNA digests (see arrows in Figure 4). The band in each case is the one known to contain the regulatory DNA, and the mobility shift indicates that small deletions of regu-

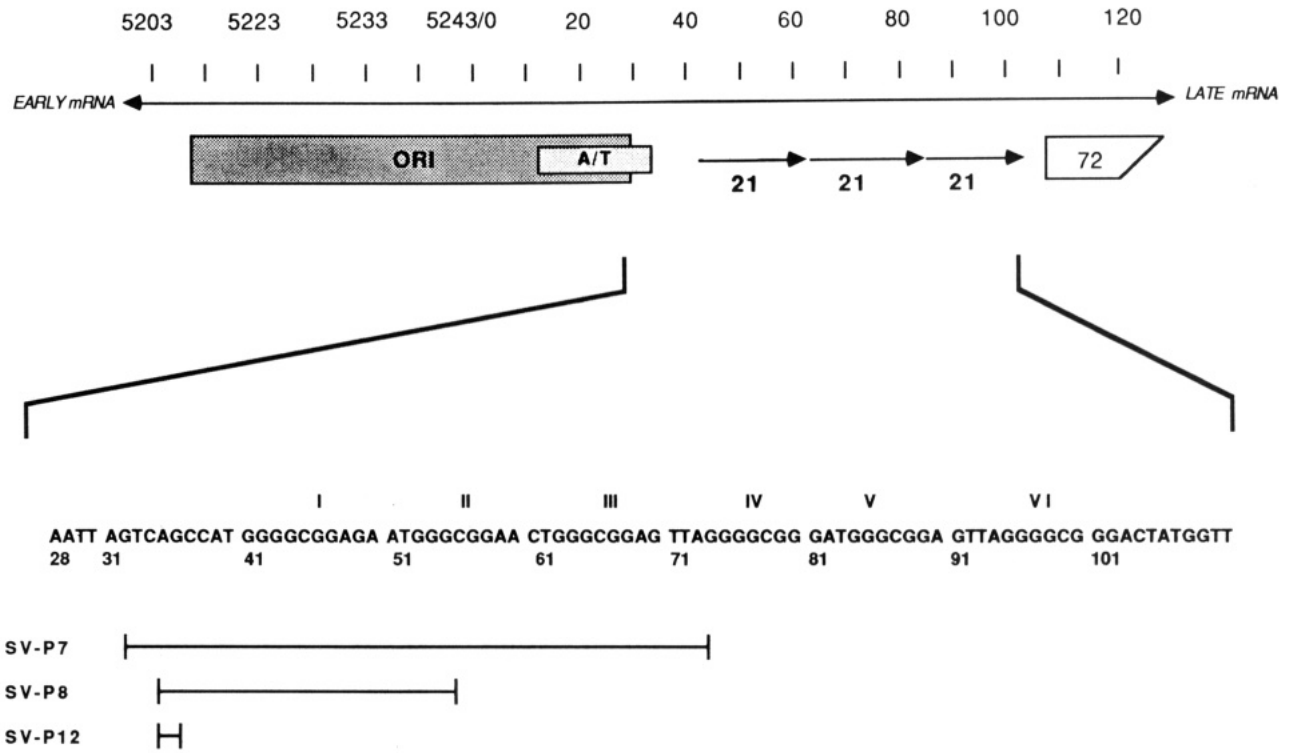

FIGURE 5: DNA sequence of mutant viruses isolated from cisplatin-resistant, SV40-infected cell lysates. Above is shown the SV40 control region. The bidirectional arrow designates the linear SV40 DNA sequence with nucleotide numbering as in Tooze (1981). The directions of early and late transcription are indicated. Relevant SV40 control regions are shown. Three arrows labeled "21" depict the six GC-box regulatory elements. The wedge labeled "72" is the origin proximal portion of the 72-bp viral enhancer element. Ori refers to the origin of replication. At the bottom of the figure, the SV40 GC-box region is expanded to show the nucleotide sequence from NT28-111. Within this region, the GC boxes are numbered with Roman numerals according to convention. The mutant viruses are missing nucleotides 32–73 (SV-P7), 36–56 (SV-P8), and 34–35 (SV-P12), inclusive.

latory DNA occurred in viruses SV-P7 and SV-P8.

The entire regulatory region of SV-P7, SV-P8, and SV-P12 (from nt 5170 to nt 350) was sequenced to establish the precise nature of the mutations in this region. The results showed that SV-P7 and SV-P8 had sustained small deletions in the regulatory DNA region (see Figure 5). SV-P7 has deleted GC boxes I–III and nine adjacent nucleotides. SV-P8 has deleted GC box I, half of GC box II, and seven adjacent nucleotides. The nonresistant virus SV-P12 was shown to be missing only two base pairs in the adjacent region, leaving the GC boxes intact. Aside from these deletions, no other changes occurred in the 400-nt regulatory region or in the 38 GG sequences scattered around the genome that were assayed by restriction analysis (Figure 4).

Further analysis by cleavage with the multicut restriction enzymes *Ava*II, *Stu*I, *Sau*96I, and *Hin*dIII showed that all these enzyme recognition sequences were intact; in each case, agarose gel electrophoresis showed patterns identical with that of wild type, except that small fragments containing the origin had increased mobility, as expected.

Drug-Resistant Viruses Produce Normal Amounts of DNA with Enhanced Function. Cisplatin is known to inhibit SV40 DNA syntheses strongly (Ciccarelli et al., 1985), and DNA replication has been proposed as the most likely target of drug action [see Sherman and Lippard (1987) and Nicolini (1988)]. Therefore, it seemed possible that mutants SV-P7 and SV-P8 might produce higher levels of DNA when challenged by drug. This possibility must be tempered somewhat since deletions in SV40 GC boxes generally led to lower levels of DNA in the absence of drug [reviewed by DePamphillis (1988)], and this was confirmed for SV-P7 and SV-P8 (Buchanan & Gralla, 1990). Nevertheless, the production of DNA by the three viruses was measured in the presence of drug.

CV-1 monolayers infected with SV-P7 and SV-P8 viruses were challenged with 15 μM cisplatin, the medium changed at 8 h, and DNA was harvested at various times from 30 to 72 h postinfection. Figure 6 shows the amount of SV40 DNA produced during this time. The resistant viruses SV-P7 and SV-P8 produce about half the amount of DNA the wild type does. This is proportional to the lowered amount of DNA they produce during drug-free infections (Buchanan & Gralla, 1990). Therefore, the enhanced production of viable virus by these mutants is not due to the production of more DNA, since less is actually produced. We conclude that the DNA produced by the mutant in the presence of cisplatin is more functional; more viable virus is produced from less DNA.

Quantitatively, these results show that the mutants produce approximately 3 times the number of viable viruses from half the DNA. This occurs under conditions of severe drug challenge, where the yield of wild-type virus is reduced by 95–99%. It is not known how the loss of regulatory DNA allows the DNA to function more effectively under these conditions, and some possibilities will be discussed below.

Discussion

Serial growth of simian virus 40 in the presence of increasing amounts of the antitumor drug cisplatin led to the isolation of mutant viruses. These viruses produced, upon infection of CV-1 cells, approximately triple the number of viable viruses compared to wild type when challenged with the high amounts of drug used in the selection. The ability of the mutant viruses to resist cisplatin inhibition of growth was not due to increased production of viral DNA, since less DNA was actually produced from the mutants. Instead, the mutants must produce DNA that functions more effectively in producing viable virus

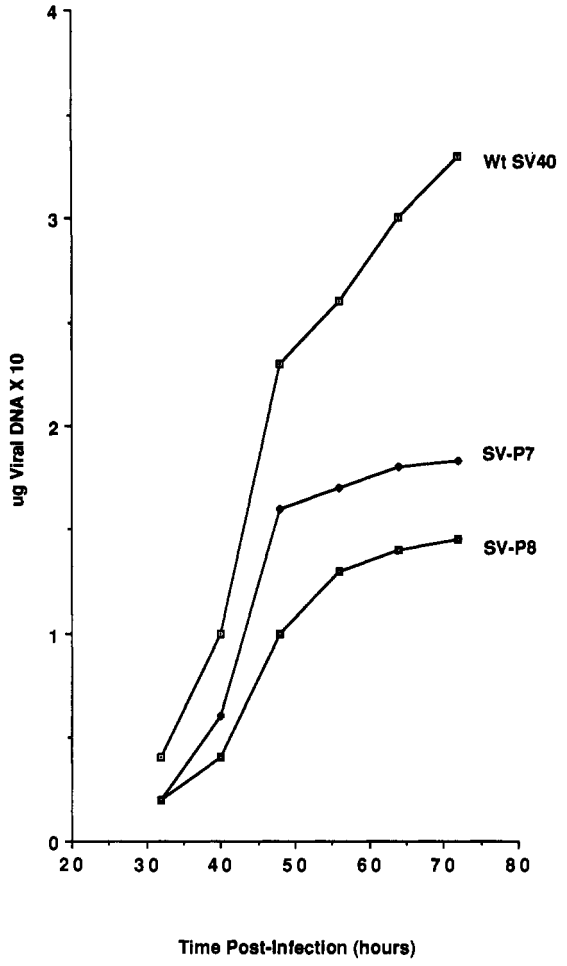

FIGURE 6: Wild-type SV40, SV-P7, and SV-P8 DNA accumulation from 30 to 72 h after exposure to 15 μM cisplatin. CV-1 cells were infected with 1–5 pfu/mL wild-type SV40, SV-P7, or SV-P8 and challenged with 15 μM cisplatin for 8 h, and the DNA was harvested at the indicated times. Purified DNA was linearized, electrophoresed on a 1% agarose gel with known amounts of SV40 DNA, stained with ethidium bromide, and photographed. The quantity of DNA in each lane was determined by densitometry of underexposed film.

than wild type after exposure to cisplatin. The two mutants isolated had sustained small deletions within and adjacent to the viral GC boxes, a regulatory element shown previously to be a strong target of cisplatin attack in vitro (Gralla et al., 1987). GC boxes influence both replication and transcription of SV40 [see DePamphillis (1988)], and multiple copies of these elements occur in the exposed regulatory region flanking several oncogenes (Dynan & Tjian, 1985). Overall, these results further the possibility that attack on GC-box DNA mediates part of the antitumor activity of cisplatin, a hypothesis that has significant implications for drug design [see the introduction and Gralla et al. (1987)].

The use of DNA viruses to study anticancer drug resistance has not been reported previously, and it is difficult to make a quantitative comparison to other studies. Most previous studies involve the selection of drug-resistant cell lines (Gross et al., 1986; Richon et al., 1987; Teicher et al., 1987; Eastman & Schulte, 1988; Nicolini, 1988). In those cases, resistance is defined quantitatively by cell killing curves. Levels of resistance in such cell lines range from less than 2-fold to up to a 100-fold. Although several reasonable possibilities for resistance have been raised (see the introduction), none are proven, and their cumulative effect seems inadequate to account for the observed resistance [see Nicolini (1988), for example]. No gene amplification has been observed in cisplatin-resistant cells, leaving the lesions responsible for resistance unknown. These viral data raise the new possibility that some tumors and cells may be resistant because they have lost critical DNA targets that include strings of guanosines and that this loss could have contributed to resistance.

The current results also bear on issues related to the mechanism of action of cisplatin and certain alkylating drugs (Gralla et al., 1987). In general, these antitumor drugs are thought to work by cross-linking DNA at relatively random locations and inhibiting DNA synthesis. These experiments confirm (Ciccarelli et al., 1985) that in the SV40 in vivo test system DNA synthesis is strongly inhibited by cisplatin. However, DNA synthesis is comparably inhibited in wild-type viruses and those with GC-box deletions, yet the mutants produce 3-fold more viable virus from half the number of DNA molecules synthesized in the presence of cisplatin. It is not yet known why, in cisplatin-challenged infected cultures, the mutant DNA is more functional than wild type, but several possibilities exist.

One possibility relates to a specific feature of the SV40 life cycle. In addition to controlling transcription and replication levels, the GC boxes I–III also influence SV40 DNA replication timing somewhat. In the absence of drug, these viruses begin replication 1–4 h earlier than wild type (Buchanan & Gralla, 1990), an observation confirmed in the presence of drug (not shown). Since the formation of cisplatin cross-links is a two-step process in which monoadducts are formed initially and convert to toxic bifunctional adducts over several hours (Zwelling et al., 1981), it is possible that early DNA production may be advantageous. Therefore, it is possible that the DNA produced at this time will be more functional since the effects of cisplatin on cell metabolism may not have yet reached their maximum inhibitory phase. In this scenario the mutant viruses are resistant since they have altered replication timing to allow more DNA to be produced at a critical, very early time when the inhibition by drug may be less severe.

The alternative, more interesting, possibility is that the viruses are resistant because they have lost a target for drug-induced inhibition of function. This would allow the DNA made in the presence of cisplatin to function more effectively. In this scenario the drug-challenged mutant templates could produce functional late transcript more efficiently or could be appropriately packaged into virus more easily or could produce more functional early transcript upon reinfection of new cells. At this time it is not possible to distinguish among these and other possibilities. These distinctions, however, are important because they bear on whether GC boxes and possibly other G-string sequences are functional targets of attack by a wide variety of antitumor drugs (Gralla et al., 1987; Mattes et al., 1988). This suggestion in turn has important implications for rational design of new platinating and alkylating antitumor drugs that attack such sequences with greater selectivity.

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Synthesis and Distribution of Primer RNA in Nuclei of CCRF-CEM Leukemia Cells†

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ABSTRACT: The distribution of primer RNA and RNA-primed nascent DNA in nuclei of CCRF-CEM leukemia cells was examined, and the primer RNA purified from the nuclear matrices of these cells was characterized. RNA-primed nascent DNA was radiolabeled by incubating whole-cell lysates with [α-^{32}P]ATP and [^{3}H]dTTP in the presence of approximately physiological concentrations of the remaining ribo- and deoxyribonucleoside triphosphates. The primer RNA was purified by cesium chloride density gradient centrifugation and analyzed by polyacrylamide gel electrophoresis. Nuclear subfractionation studies revealed that at least 94% of the primer RNA and RNA-primed nascent DNA were located within the insoluble matrix fraction of the nucleus. The predominant primer RNA isolated from the nuclear matrix was 8-10 nucleotides in length, and several lines of evidence indicated that this oligoribonucleotide was the functional primer RNA. Essentially all of the matrix primer RNA was covalently linked to the newly replicated DNA as demonstrated by its buoyant density in cesium chloride gradients, phosphate-transfer analysis, and sensitivity to DNase I. Analysis of ^{32}P transfer from [α-^{32}P]dTTP revealed a random distribution of ribonucleotides at the 3′-end of the primer RNA. Data obtained from mixing experiments indicated that the association of RNA-primed nascent DNA with the nuclear matrix was not the result of aggregation of these fragments with the nuclear matrix. No significant amount of either primer RNA, RNA-primed nascent DNA, or phosphate transfer was detected in the high-salt-soluble (nonmatrix) fraction of the nucleus, although the nonmatrix fraction contained most of the newly replicated DNA. These observations provide evidence that in CCRF-CEM cells the synthesis of both primer RNA and RNA-primed Okazaki fragments takes place on the nuclear matrix, and that the primer RNA is degraded prior to the migration of the nascent DNA away from the matrix-bound DNA replication sites.

The discontinuous synthesis of DNA (Okazaki) fragments on the lagging strand of the replication fork is an important biochemical process involved in DNA replication. Most of the Okazaki fragments that are recovered from eukaryotic cells contain on oligoribonucleotide primer that is covalently attached to the 5′-end of the fragment (Waqar & Huberman, 1975a,b; Tseng & Goulian, 1977; Tseng et al., 1979; Kitani et al., 1984). This primer RNA, which is about 10 nucleotides in length (Tseng & Goulian, 1977; Tseng et al., 1979; Kitani

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