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Effects of Incorporation of 6-Thioguanine into SV40 DNA

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SUMMARY

The antileukemic agent 6-thioguanine (TG) is thought to inhibit DNA synthesis as a result of its incorporation into DNA. In the present study we have examined the nature of this inhibition, using replication of SV40 viral DNA as a model system. Addition of TG to SV40-infected CV1P cells from 22 to 24 hr post infection causes a dose-dependent inhibition of viral DNA synthesis. This inhibition plateaus between 250 and 2500 μM TG, resulting in a maximum decrease of viral DNA synthesis of about 50%. Pulse-chase experiments showed no detectable slowing of elongation of nascent DNA chains, whereas measurement of the conversion of incorporated ^{3}H-dThd into supercoiled viral DNA suggested that elongation might be slightly inhibited, but by no more than 20%. Since inhibition of elongation could not account for the total depression of DNA synthesis, we hypothesized that inhibition of initiation of DNA replication takes place. This hypothesis was tested by radioactively labeling newly synthesized viral DNA and then assessing the ability of these molecules to reenter the replicating pool by density labeling with bromodeoxyuridine. The fraction of TG-containing molecules able to re-initiate replication was decreased 15%, compared to control. This effect, which was dependent on the concentration of TG added to the medium, was closely correlated to the extent of TG incorporation into the viral genome. We concluded that a portion of SV40 viral DNA synthesis inhibited by TG is due to an effect on initiation, and hypothesized that this effect may be caused by the substitution of TG for guanine in critical recognition sequences at the origin of replication. We proceeded to test this hypothesis by constructing SV40 origin sequences containing TG and then measuring their ability to bind T-antigen *in vitro*. The necessary deoxynucleoside triphosphate, TdGTP, was obtained by chemical phosphorylation of thiodeoxyguanosine. In order to selectively place TG within the desired region, a plasmid containing the T-antigen binding sequences was linearized so as to place these sequences at one end of the molecule, and then digested briefly with exonuclease III. The excised strand was resynthesized by use of the Klenow fragment of DNA polymerase I along with various nucleotide mixtures. Although resynthesis with mixtures containing TdGTP in place of dGTP was impeded somewhat, it was possible to achieve complete resynthesis with this analog. We found that incorporation of TG into SV40 DNA in this manner resulted in a drastic decrease in two sequence-specific functions, namely, cleavage by the restriction endonuclease Bgl I and binding to immunoaffinity-purified T-antigen. We suggest that the consequences of TG incorporation may depend on the sequence in which that incorporation takes place, and that a basis for some of the delayed effects in cells exposed to TG may be the disturbance of interactions between proteins and specific DNA sequences.

Several anticancer and antiviral agents are purine or pyrimidine analogs which are thought to exert their actions as a result of incorporation into nucleic acids. For some of these agents, especially ones with modified sugar moieties such as arabinosylcytosine, it is evident that incorporation of analog into the nascent strand of DNA can pose an immediate obstacle to replication, manifested as slowing and termination of chain elongation (1-3). Although it is not entirely clear how these lesions ultimately lead to cell death, the characterization of these initial effects provides a basis for the formulation of hypotheses to explain how such agents cause cytotoxicity.

The consequences of incorporation of certain other analogs such as TG are even less clear, however. Although incorporation of TG into DNA has often been proposed as the effect responsible for this drug's cytotoxicity (4, 5), the mechanism by which TG incorporation leads to cell death has remained obscure. One reason for this is that the incorporation of the drug per se produces no immediate effects on DNA synthesis (6). Rather, there is a considerable delay between incorporation and eventual arrest of cells in the late S/G2 phase of the subsequent cell cycle (6, 7). We have previously shown that this delayed arrest appears to be due to gross chromosomal disruption related to the presence of TG in the template strand of replicating DNA

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ABBREVIATIONS: TG, 6-thioguanine; PI, post infection; BrdUrd, 5-bromo-2′-deoxyuridine; FdUrd, 5-fluoro-2′-deoxyuridine; T-ag, T-antigen; HU, Hydroxyurea; TdGTP, thiodeoxyguanosine 5′-triphosphate; SV40, simian virus 40; EtOH, ethanol; EDTA, ethylenediaminetetraacetate; HPLC, high performance liquid chromatography; Exo III, exonuclease III.

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(8, 9), and our more recent efforts have been aimed at understanding the mechanism by which incorporated TG disturbs subsequent rounds of replication.

In the first part of this study we investigated the effects of TG on replication of SV40 DNA in intact, infected cells. This system was chosen because it represents one of the best characterized models for eukaryotic replication currently available. One unexpected result of these experiments was that extensive incorporation of TG into viral DNA produced a relatively minor inhibition of net viral DNA synthesis, suggesting that the gross extent of TG incorporation may not be a reliable determinant of biological effect. Second, a major portion of the DNA synthesis inhibition we did observe was related to inhibition of initiation of new rounds of replication. Since it is well known that initiation of SV40 replication is critically dependent upon binding of the viral gene product T-ag to the origin of replication, we hypothesized that the presence of incorporated TG in T-ag-binding sites may disrupt this binding interaction. In the second part of this study we tested this hypothesis by constructing TG-containing SV40 origin sequences and measuring their ability to bind isolated T-ag.

Materials and Methods

An aliquot of plaque-purified SV40 virus, strain 776, was a gift from M. Konig (National Institutes of Health), as was a sample of the host cell line, CV1P. Cells were grown in alpha-minimal essential medium (GIBCO) supplemented with 5% fetal bovine serum (Biofluids Inc., Rockville, MD) at 37° in a humidified 5% CO_2 atmosphere. Infections were performed using 5 plaque-forming units/cell in medium containing 2% serum. *methyl*-^{3}H-dThd (50 Ci/mmol) and 2-^{14}C-dThd (56 mCi/mmol) were supplied by Moravek Biochemicals (Brea, CA). All other chemicals were of analytical reagent quality. Centrifugations were performed in a Beckman L8-70M ultracentrifuge and spectral data were obtained using a Perkin-Elmer Lambda-3B spectrophotometer interfaced with model 3600 data station.

Inhibition of viral DNA synthesis. In initial experiments, cultures were treated during various intervals PI with either 2.5 mM TG or control vehicle. Cells were then washed free of drug and exposed to medium containing ^{3}H-dThd (1 μCi/ml) for 30 min, after which the medium was removed and lysis was performed by the method of Hirt (10). Lysate was scraped into a tube and made 1 M in NaCl, gently mixed, and then stored at 4° overnight. Cellular DNA was pelleted by centrifugation at 27,500 × *g* for 1 hr. An aliquot of the supernatant was then acidified, and precipitated viral DNA was collected on glass fiber filter papers, which were then washed, dried, and assayed for ^{3}H by liquid scintillation counting. In later studies the labeling was performed with medium containing 1 μM FdUrd + 5 μM dThd (2 μCi/mmol), in order to avoid effects on ^{3}H incorporation by drug-induced perturbation of *de novo* nucleotide synthesis (11).

Elongation of nascent SV40 DNA. Cultures were treated at 22 hr PI with either medium containing 2.5 mM TG or control medium. Two hr later the dishes were washed extensively and the 5′ labeling period was begun with either ^{14}C-dThd (1 μCi/ml, control dishes) or ^{3}H-dThd (10 μCi/ml, TG dishes). At the end of 5 min the dishes were washed twice with medium containing 1 μM cold dThd and chased for the times indicated, after which Hirt lysis was performed. Hirt supernatant was extracted with $HCCl_3$ and nucleic acids were then precipitated with EtOH. After spinning down the precipitate and draining off EtOH, DNA was dissolved in a small volume of TE buffer (10 mM Tris, 1 mM EDTA), pH 7.5, and control and TG samples from each time point were combined and sedimented through a 5–20% neutral sucrose gradient in an SV41 rotor at 28,000 rpm, 4° for 17 hr. Gradients were fractionated by pumping from the bottom. After correction for quenching and spillover, radioactivity in each fraction was calculated as the percentage of total cpm recovered in each gradient. A more quantitative approach was also used, in which we determined the fraction of incorporated ^{3}H-dThd converted to form I (supercoiled) DNA in 30 min (12). Cells were infected as above and then treated with 2.5 mM TG, 0.1 mM HU, or 0.2 mM HU. These two concentrations of HU inhibited viral DNA synthesis by 30% and 59%, respectively (data not shown). After drug treatment, cells were washed and exposed to medium containing ^{3}H-dThd (2.5 μCi/ml) for 30 min at which time cells were lysed. Since the effects of HU may be rapidly reversible, HU was included during the labeling period at the same concentration as during the pre-labeling period. Viral DNA was prepared as above and Form I was isolated by sedimentation through alkaline sucrose gradients (12). After fractionation and determination of acid-precipitable radioactivity, the portion of recovered cpm appearing in the form I peak was determined. To correct for variability between gradients, each gradient contained ^{3}H-labeled experimental DNA plus ^{14}C-labeled control DNA. Data are therefore expressed as fraction of ^{3}H recovered as form I/fraction of ^{14}C recovered as form I.

Reentry of SV40 DNA molecules into the replicating pool. Infected cells were treated at 22 hr PI with TG or control medium, each containing 1 μCi/ml ^{3}H-dThd, for a 2-hr period. After drug and label were washed out, chase medium containing 50 μM BrdUrd + 20 μM FdUrd + 9 μM dCyd was added (13). Incubation in chase medium was continued for 8 hr, at which time Hirt lysis, extraction, and EtOH precipitation were performed. The samples were then centrifuged for 24 hr in a CsCl solution (starting density = 1.72) in a half-filled Ultra-Clear tube, in a type 70.1 rotor at 40,000 rpm, 20°. Tubes were punctured at the bottom and five-drop fractions were collected and assayed for radioactivity. We observed two clearly separated peaks in each sample, corresponding to DNA having native density and to DNA completely substituted in one strand with BrdUrd. The fraction of radioactivity found in the more dense band in each gradient was determined and plotted as a function of TG concentration.

Incorporation of TG into SV40 DNA. Infected cells were exposed to various concentrations of TG from 22 to 46 hr PI, after which they were washed and lysed. The Hirt supernatant was extracted twice with $HCCl_3$ and DNA was then precipitated with ethanol, redissolved in TE buffer, and centrifuged in a solution of CsCl (1.55 g/ml) and ethidium bromide (80 μg/ml) in TE at 50,000 rpm in a type 70.1 rotor, at 4° for at least 20 hr. The more dense band, containing closed circular DNA, was withdrawn into a 1-ml syringe and extracted four times with water-saturated butanol. CsCl was removed by processing the sample through four cycles in a Centricon 30 concentrator (Amicon Corp.). Viral DNA was diluted with TE to a concentration of 0.50 A_{260} units/ml and then scanned for absorbance from 380 to 320 nm, to detect incorporated TG.

Synthesis of TdGTP. The starting material, thiodeoxyguanosine (a generous gift from Dr. L. B. Townsend), was dissolved in trimethyl phosphate (20 mg/250 μl) and chilled to −5°. To this solution was added 15 μl of distilled $POCl_3$, and the mixture was allowed to stir at −5° for 3 hr. At this time 2 μl of the reaction mixture were treated with 2 μl of triethylamine and analyzed by anion exchange HPLC to confirm the formation of TdGMP (14). The remainder of the reaction mixture was slowly added to 700 μl of tris (tributylammonium) pyrophosphate and allowed to stir at −10° for 3 hr. The reaction mixture was slowly neutralized with cold triethylamine (~400 μl) and lyophilized (15). The residue was dissolved in 1 ml of H_2O and chromatographed on a 2 × 20 cm DEAE-cellulose column (HCO_3^- form) using a linear gradient up to 0.4 M triethylammonium bicarbonate (pH 7.5). Appropriate fractions containing TdGTP (90–99% by HPLC) were combined and evaporated to dryness. Excess triethylammonium bicarbonate was removed by addition and evaporation of ethanol. The purity of the product was assessed by anion exchange HPLC; using an Altex SAX column and a mobile phase of 200 mM sodium phosphate at 2 ml/min, the product had a retention time of 10.5 min. An overall yield of 20% was obtained.

Plasmid DNA. The plasmids pJLO and pKP45, generous gifts from Dr. J. Li, Johns Hopkins University, were propagated in *Escherichia*

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coli strain HB101. pKP45 was derived from pBR322 by deletion of nucleotides 677–2364, and pJLO was obtained by replacing the *Hind III/Sph I* fragment of pKP45 with the *Hind III/Sph I* portion of the SV40 genome (16). Plasmid DNA was isolated by standard techniques (17).

Incorporation of nucleotides into SV40 origin sequences. The overall scheme for this procedure is shown in Fig. 1. Plasmid DNA was first linearized with 3–5 units/μg *Hind III* (Boeringer Mannheim Biochemicals) in 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 50 mM NaCl. The 3′ ends of the linear molecule were then excised by treatment with Exo III (Bethesda Research Laboratories) at a concentration of 16 units/μg DNA, in 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, and 10 mM β-mercaptoethanol at 23°. The length of incubation was an experimental variable. After extraction with $HCCl_3$ and EtOH precipitation, an aliqout (1.5–2 μg) of the product of the Exo III reaction was usually digested with 10 units of S1 nuclease (Bethesda Research Laboratories) in 50 mM NaOAc (pH 4.6), 200 mM NaCl, 100 mM $ZnSO_4$, and 0.5% glycerol, in order to remove the single-stranded tails and permit an estimation of the size of the resulting attenuated duplex by agarose electrophoresis. Resynthesis of the excised portion of the linearized plasmid was accomplished by incubation with the Klenow fragment of DNA polymerase I (International Biotechnologies, 2 units/μg DNA) in a buffer containing 32 mM Tris-HCl (pH 8.4), 167 mM KCl, 3 mM $MgCl_2$, 1.3 mM dithiothreitol, and nucleotide mixtures as indicated.

Evaluation of completeness of resynthesis. In most resynthesis reactions α-^{32}P-dCTP (Amersham) was included at a final specific activity of 12 μCi/nmol. Following resynthesis the reaction mixture was extracted once with $HCCl_3$, and unincorporated radioactivity was removed by three cycles of concentration and dilution with TE, pH 8.0, using a Centricon 30 microconcentrator (Amicon). The sample was again diluted with TE and DNA was determined by UV absorbance. Radioactivity was assayed by liquid scintillation counting in order to calculate the specific activity of the products, which was usually on the order of 1000 cpm/ng. An aliquot of the resynthesized DNA was then digested with Sph I and the migration of the resultant fragments on an 8% denaturing polyacrylamide gel was compared to end-labeled standards.

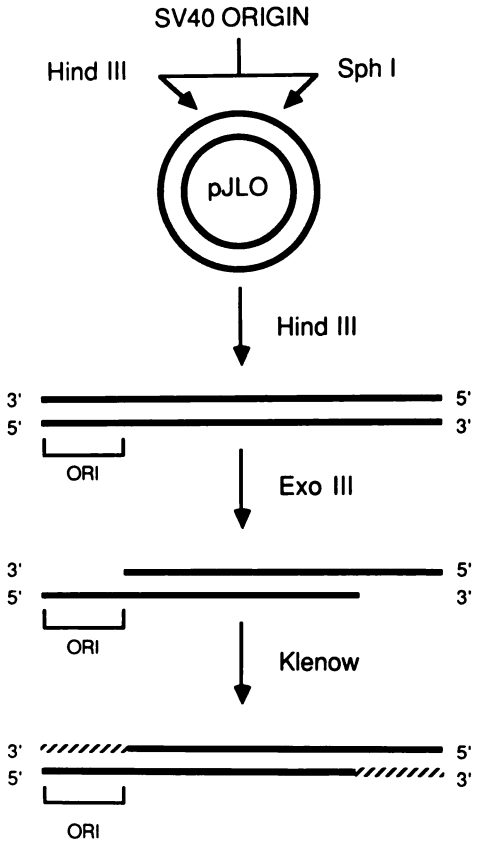

Fig. 1. General scheme for incorporation of TG into SV40 origin sequences. Circular pJLO was linearized with *Hind III*, placing the origin region at one end of the molecule. Controlled digestion with Exo III removed about 200 bases from each 3′ end. These portions were then resynthesized with the desired nucleotide mixture.

Isolation of SV40 T-antigen. The procedure of DiMaio and Nathans (18) was followed, in which CV-1 cells were infected with the T-ag-overproducing mutant strain cs1085, and then extracted as previously described (19). T-ag was isolated from this extract by use of an immunoaffinity column prepared by crosslinking PAb419 monoclonal antibodies (see Ref. 20, obtained from ascites fluid) to Protein A Sepharose (Pharmacia) with dimethyl pimelimidate (21). The product was analyzed by sodium dodecyl sulfate-polyacrylamide electrophoresis and found to migrate as a single band of about 90 kDa, identical to a sample of T-ag provided by S. Smale, University of California.

T-ag binding assays. Conditions for filter binding assays were taken from the report by Fisher *et al.* (22). Varying amounts of ^{32}P-DNA (0–16 ng) were incubated with 0.03 μg of T-ag in a total volume of 100 μl of binding buffer (20 mM Na_2HPO_4, pH 7.0, 0.5% Nonidet P-40, 3% dimethyl sulfoxide, 0.01% bovine serum albumin, 0.1 mM EDTA, 2 mM dithiothreitol, 150 mM NaCl) at room temperature for 1 hr. Samples were then applied to a 24-mm nitrocellulose filter (BA85, Schleicher and Schuell) and washed with two 100-μl aliquots of washing buffer (binding buffer minus Nonidet P-40, NaCl, and DMSO). Filters were air-dried and assayed for radioactivity by liquid scintillation counting.

Results

Inhibition of SV40 DNA synthesis by TG. Preliminary studies were performed to determine the appropriate length and timing of TG treatment to obtain maximal inhibition of viral DNA replication. In these experiments we used incorporation of ^{3}H-dThd as an approximation of DNA synthesis rate. We found that inhibition caused by 2-hr treatments was not substantially augmented by longer incubation times, and that treatments near the beginning of the DNA synthesis phase of infection (22 hr PI) were no more effective than those applied near the end of that phase (40 hr PI). Incorporation of ^{3}H-dThd into viral DNA was maximally inhibited, by about 50%, by 2 hr exposure to 2500 μM TG. A more reliable picture of the effects of TG on DNA synthesis was obtained by applying the method of Naaktgeboren *et al.* (11) in which FdUrd is used to eliminate *de novo* dTMP synthesis, leaving exogenously added dThd as the only source of dThd nucleotides. The specific activity of the dTTP pool is therefore unaffected by changes in endogenous nucleotide metabolism. Fig. 2 summarizes the results of several such experiments, each point representing the mean of three to five determinations on a given day. Inhibition of viral DNA synthesis by TG began in the low μM range and increased until an apparent maximum of about 50% was reached, at mM concentrations of TG.

Effects of TG on elongation of nascent SV40 DNA. Replication of SV40 DNA involves the conversion of a form I (supercoiled) molecule with a sedimentation coefficient of 21 S (neutral pH) to replicative intermediates, whose sedimentation coefficients increase from 21 S to 25 S as replication approaches completion (23). The time required for completion of one replication cycle is about 15 min. We first assessed progression of initiated molecules to completion (i.e., elongation) by providing a pulse of labeled dThd followed by a chase with cold dThd for various periods. The results of these experiments are presented in Fig. 3. Under the conditions used here, form I DNA should peak at about fraction 8 (21 S), whereas replicative intermediates peak at about fraction 4–5 (25 S). As the length of the chase period increases, so does the fraction of label sedimenting as complete, form I molecules. If elongation of nascent DNA chains were inhibited by TG, one would expect to see a decrease in formation of 21 S material and a concomitant increase in ^{3}H sedimenting between 21 S and 25 S. No consistent

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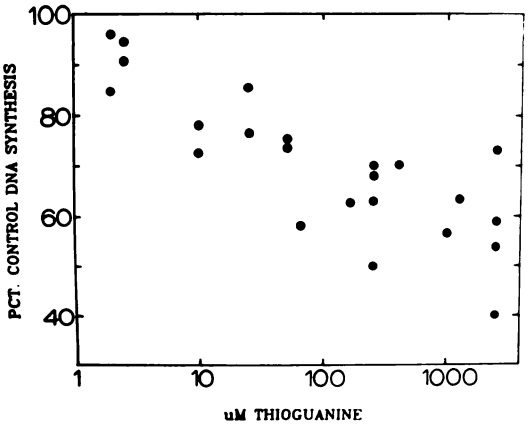

Fig. 2. Inhibition of viral DNA synthesis by TG. CV1P cells infected with SV40 were treated with various concentrations of TG from 22 to 24 hr PI, followed by 30 min exposure to ^{3}H-dThd + FdUrd. Cultures were then Hirt-lysed, and acid-precipitable radioactivity in aliquots of Hirt supernatants from these cultures was determined. Each *point* represents the mean of three to five determinations from a single experiment.

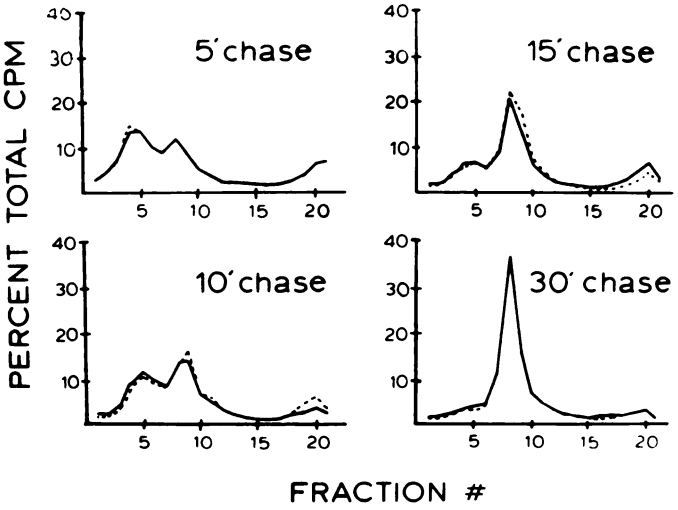

Fig. 3. Effect of TG on elongation of nascent SV40 DNA. SV40-infected cells were treated with 2.5 mM TG from 22 to 24 hr PI, then washed free of drug and exposed to ^{14}C-dThd (control) or ^{3}H-dThd (TG) for 5 min followed by incubation with unlabeled dThd for the indicated periods. Viral DNA was isolated and analyzed by neutral sucrose gradient sedimentation. Radioactivity in each fraction is expressed as a percentage of the total cpm recovered in each gradient (—, control; ---, TG treatment). These data are from one of two experiments which yielded virtually identical results.

difference between control and TG samples was detectable in this study. A more quantitative analysis of conversion of nascent DNA into form I, using alkaline sucrose sedimentation, is presented in Table 1. HU was used in these studies as a positive control, since it has been shown to inhibit SV40 DNA synthesis by a specific effect on elongation (12). Over the range of HU concentrations used there appears to be a linear relationship between inhibition of total DNA synthesis and inhibition of conversion cpm to form I. Exposure to 2.5 mM TG produced a rate of conversion to form I which was intermediate between control and 0.1 mM HU. Using the observed variance for determination of conversion to form I following treatment with 2.5 mM TG, the smallest decrease in this parameter which would be significant at the 95% confidence level would correspond to an elongation rate 80.4% of control. Although the high degree of variability inherent in making these measurements limits the detection of small changes in elongation rate, it is clear that the degree to which 2.5 mM TG inhibits conversion

TABLE 1

Inhibition of conversion of incorporated cpm into SV40 form I DNA

Infected cells were treated as indicated for 2 hr, followed by 30 min exposure to ^{3}H-dThd, lysis, and isolation of form I by alkaline sucrose sedimentation. ^{14}C-labeled SV40 DNA was added to each gradient tube to control for differences in sedimentation or recovery between individual tubes. Data are normalized to the percentage of ^{14}C-cpm recovered as form I in each tube ± standard error.

Treatment	Conversion of incorporated ^{3}H-cpm to form I (% of ^{14}C-control)	Net viral DNA synthesis (% of control)
No drug	99.6 ± 2.7 (n = 5)	100
0.1 mM HU	92.4 ± 1.6 (n = 9)[a]	70
0.2 mM HU	81.5 ± 1.3 (n = 6)[a]	41
2.5 mM TG	95.0 ± 1.3 (n = 9)	56

[a] Significant difference from "no drug" treatment ($p < 0.05$, two-tailed t test).

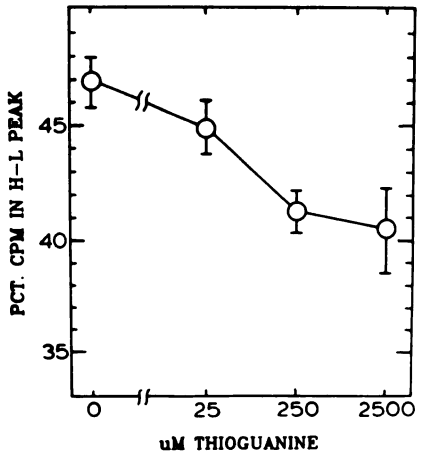

Fig. 4. Effect of TG on reentry of SV40 DNA molecules into the replicating pool. Infected cells were exposed to various concentrations of TG + ^{3}H-dThd from 22 to 24 hr PI, then changed to medium containing 50 μM BrdUrd + 20 μM FdUrd + 9 μM dCyd for 8 hr, at which time viral DNA was isolated and analyzed by CsCl equilibrium sedimentation. Gradients were fractionated, and the percentage of total radioactivity recovered appearing in the hybrid-density (*H-L*) peak (representing the DNA molecules which had re-initiated replication in the presence of BrdUrd) is plotted as a function of TG concentration. For each drug concentration the value given is the mean of three to five determinations ± standard error. Results obtained using 250 and 2500 μM TG are each significantly different from control ($p < 0.05$, two-tailed t test).

to form I is less than that corresponding to a 20% decrease in elongation rate.

Effects of TG on initiation of SV40 DNA synthesis. The finding that a major portion of the net viral DNA synthesis inhibition by 2.5 mM TG could not be accounted for by decreased elongation suggested to us that a large part of the inhibition of DNA synthesis by this regimen must be due to an effect on initiation of viral replication. In order to test this hypothesis the experimental approach of Roman and Dulbecco (24) was used. Viral DNA molecules were radioactively labeled with or without TG, after which those molecules able to re-initiate were density labeled with BrdUrd. Upon analysis by equilibrium centrifugation in a CsCl solution we observed two clearly separated peaks in each sample, corresponding to DNA having native density and to DNA completely substituted in one strand with BrdUrd. The fraction of radioactivity found in the more dense band in each gradient (i.e., the fraction of viral DNA molecules able to re-initiate replication) was determined, and the effect of various concentrations of TG on this fraction is plotted in Fig. 4. We observed in these experiments that there was a dose-dependent diminution of initiable SV40 DNA molecules as a result of TG treatment, reaching a plateau of 250–2500 μM, with the fraction of initiable molecules decreased about 15% from control levels.

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Incorporation of TG into SV40 DNA. The extent to which various concentrations of TG replaced guanine in viral DNA was assessed by measuring UV absorbance at 351 nm, which is characteristic of incorporated TG (25). In each case the DNA concentration in the cuvette was adjusted so that the absorbance at 260 nm was 0.50 unit. The degree of replacement resulting from each treatment was calculated, assuming 50 μg of DNA/A_{260} unit and ϵ = 15,000 for incorporated TG. These data are presented in Table 2. Incorporation of the drug was not detectable following treatment with 2.5 μM TG. At higher drug concentrations we observed a dose-dependent rise in TG incorporation which appeared to plateau at 250–2500 μM, in a manner similar to the inhibition of net viral DNA synthesis and of initiation. The maximum extent to which TG replaced guanine in these studies, about 8%, is similar to the frequency of replacement reported in vaccinia viral DNA (26).

Exonuclease III digestion and resynthesis of plasmid DNA. The time course of Exo III digestion of linear DNA has been previously studied and found to proceed synchronously at rates of 80–450 nucleotides/min at 37°, and about 10–30 nucleotides/min at 23° (27–29). At 23° we generally observed excision rates of 10–20 nucleotides/min.

The extent of Exo III digestion required in these studies is determined by the position of the T-ag-binding sites relative to the site used to linearize the plasmid. In the present case we used *Hind III* for linearization, thereby requiring digestion of about 100 nucleotides from the 3′ ends of the molecule to ensure complete exposure of the two highest affinity binding sites (see Fig. 5). We found that a 10-min Exo III treatment of 23° followed by digestion with S1 nuclease (to remove the residual single-stranded tails) usually gave products ranging in length from 2000 to 2150 bp, corresponding to digested regions of 100–175 nucleotides.

The functional characterization of our synthetic TdGTP was accomplished by testing its ability to replace dGTP as a substrate for DNA polymerase. Fig. 6 shows the absorbance spectra of Exo III-treated pJLO which had been resynthesized using either all four normal dNTPs or with a mixture containing TdGTP in place of dGTP. The peak at 351 nm is characteristic of incorporated TDGTP (25). We also obtained such spectra when using the same sets of substrates in a nick-translation reaction with calf thymus DNA, and upon HPLC analysis of the products of enzymatic hydrolysis of this material, we observed a peak with the same elution and spectral characteristics as TdGuo (not shown).

The completeness of resynthesis was assessed by cutting resynthesized molecules with Sph I and determining the length of the ^{32}P-labeled fragments by denaturing polyacrylamide electrophoresis. A time course of resynthesis is presented in Fig. 7. Fully resynthesized *Sph/Hind* fragments should run as single strands 205 bases in length, or slightly behind the 200 base fragments in the BstN I-cut SV40 DNA standards. We found that, whereas control resynthesis was complete by 5′, resynthesis with TdGTP in place of dGTP proceeded much more slowly and appeared to stall at specific loci. In the experiment shown in Fig. 7, resynthesis with TdGTP was approaching completion after 60′ of incubation. By extending the incubation time and increasing the nucleotide concentrations we were able to achieve complete resynthesis with TdGTP, as illustrated in Fig. 8.

***Bgl I* digestion of TG-containing DNA.** The effect of incorporated TG on susceptibility to digestion by the restriction endonuclease Bgl I was studied using unlabeled resynthensized DNA (Fig. 9). Since pJLO contains two recognition sites for *Bgl I*, complete digestion of *Hind III*-cut pJLO yields three fragments with lengths of 70,904, or 1373 bp. Under the conditions used, control-resynthesized DNA was completely digested by 30 min. In contrast, very little of the TG-resynthesized DNA was able to be cut to completion, even after 120 min. Instead, we found that the partial digest product had a length of 1443 bp, indicating that the enzyme was unable to cut its recognition sequence located within one of the resynthesized regions, at base number 99. The absence of full length pJLO and the presence of the 904 bp fragment indicate that the other *Bgl I* site, in the unreconstructed region, was cut normally.

T-ag binding to TG-containing SV40 origin DNA. The ability of T-ag to bind to resynthesized SV40 origin sequences was assessed in the experiment shown in Fig. 10. Whereas control-resynthesized pJLO demonstrated saturable binding with a K_d on the order of 10 pM, TG-resynthesized pJLO was bound only slightly better than pKP45 DNA (not shown), which contains no T-ag-binding sites.

TABLE 2

Replacement of Gua by TG in SV40 DNA

The extent to which TG was substituted for guanine in each of the samples was calculated assuming that 1 A_{260} unit = 50 μg of DNA, ϵ = 15,000 for incorporated TG, and the G/C content of SV40 DNA is 41%.

TG concentration	Replacement of Gua by TG in Form I SV40 DNA
μM	%
2.5	
25	1.7
250	6.4
2500	8.1

Discussion

Effects of TG treatment of intact, SV40-infected cells. Several previous experiments have indicated that the incoporation of TG into DNA is a cytotoxic event and that replacement of guanine in DNA by TG on the order of 1–2% can induce a

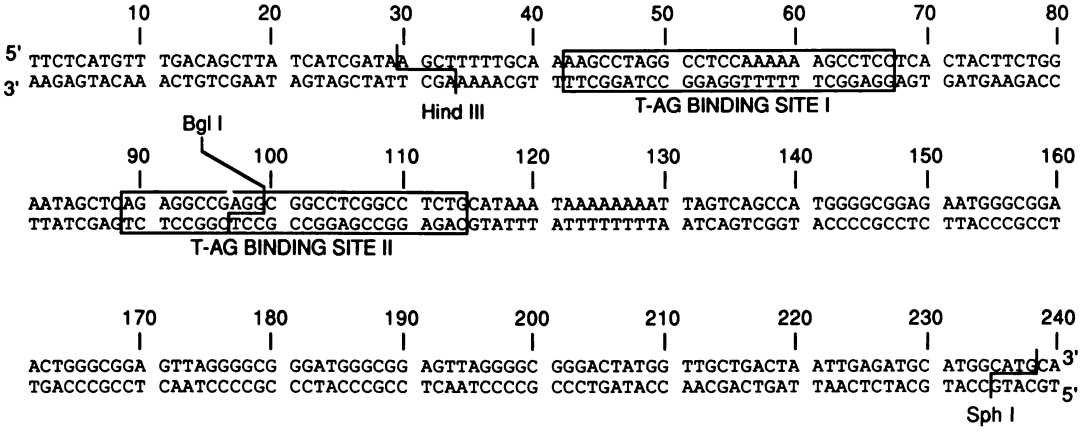

Fig. 5. A portion of the sequence of pJLO is shown, illustrating the two highest affinity T-ag-binding sites and their relationship to the *Bgl I*, *Hind III*, and *Sph I* scission loci.

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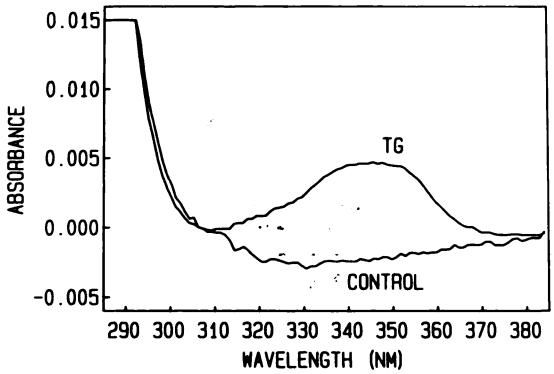

Fig. 6. Incorporation of TG into pJLO is demonstrated by an absorbance band with a peak at 348 nm. Each sample was adjusted to have an absorbance of 0.11 at 260 nm. Using the assumptions stated in Table 2, this corresponds to a net replacement of Gua by TG in 14% of the plasmid molecule. If replacement in the reconstructed regions was 100%, this would mean that about 165 nucleotides were excised and then resynthesized at each 3′ end. This is consistent with our observation that the conditions used in this experiment typically result in digestion of 100–175 nucleotides.

2–4 log decrease in the clonogenicity of mammalian cells (4, 9, 30). We were therefore quite surprised that concentrations of TG, which resulted in as much as 8% replacement of guanine, inhibited viral DNA synthesis by only about 50%. Since the replication of SV40 is thought to mimic many aspects of chromosomal replication, this suggests that the gross extent of incorporated TG is not generally related to DNA synthesis inhibition in eukaryotic cells. It is also possible that differences between the viral and chromosomal replication systems exist which account for the relative sensitivity of the latter. As discussed below, we feel that both of these interpretations may be valid.

In spite of the relatively modest magnitude of TG-induced inhibition of SV40 DNA synthesis, we attempted to define the relative contributions to this inhibition of effects on elongation and initiation, since this had not been accomplished in any system previously. At first we had expected that the primary effect of incorporated TG would be termination of nascent DNA strands, or at least a reduction in the rate of their elongation (8). For that reason the results in Fig. 2 and Table 1 were unexpected, as they demonstrate that, under conditions where net viral DNA synthesis is decreased by about 50%, elongation is not significantly inhibited. The failure of an effect on elongation to account for total inhibition of viral DNA synthesis led us to test the possibility that initiation was being inhibited by TG. We found (Fig. 3) that molecules synthesized in the presence of TG did not reenter the replicating pool as efficiently as native DNA. This effect was dependent on the concentration of TG used, resembling the dose-response relationship observed for net viral DNA synthesis inhibition (Fig. 1) and incorporation of TG into viral DNA (Table 2). Although the absolute magnitudes of these changes were small, the inhibition of re-initiation of molecules at the two highest drug concentrations was significant at the 95% confidence level. Therefore, in at least a qualitative sense, these findings demonstrate that a significant consequence of TG incorporation into SV40 DNA is inhibition of initiation.

Since SV40 replication has been extensively characterized, it is possible to formulate some specific, testable hypotheses to explain the observed inhibition of initiation by TG. Replication of the viral genome begins at a single, defined region near the

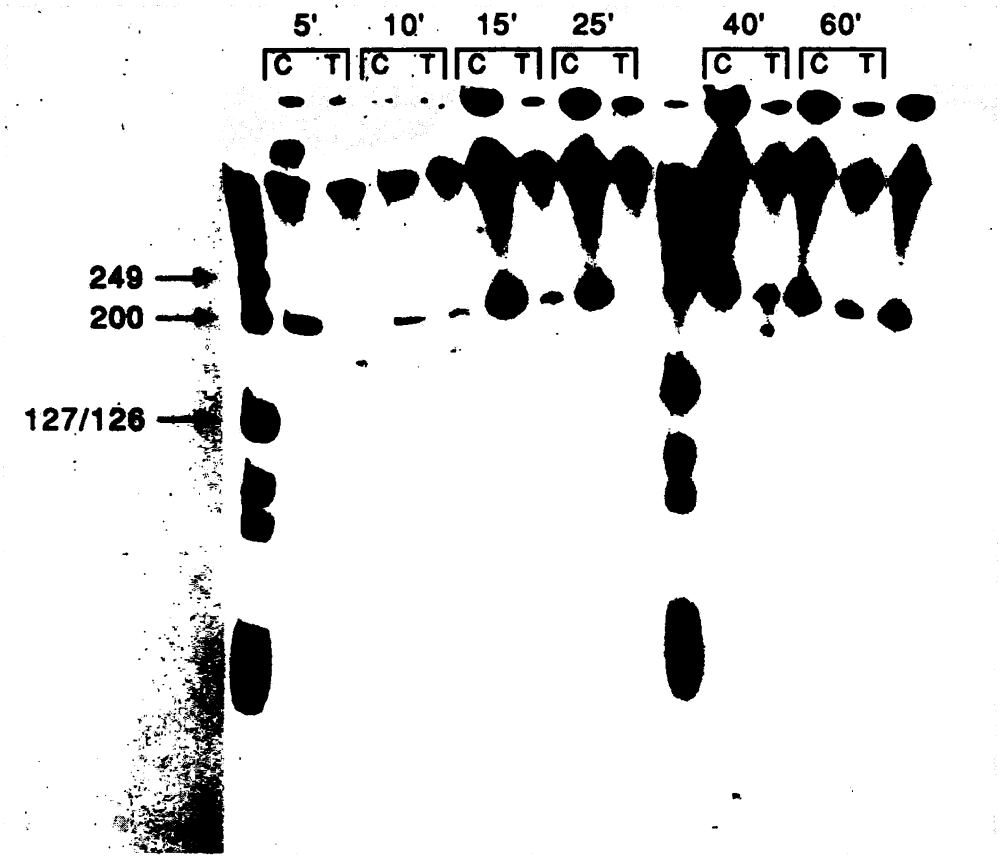

Fig. 7. A time course is shown for resynthesis of Exo III-treated pJLO with a nucleotide mixture containing 670 μM dATP, dTTP, and dCTP (including α-^{32}P-dCTP at a final specific activity of 0.25 μCi/nmol) and either 670 μM dGTP (control, *C*) or 300 μM TdGTP (*T*). At the indicated times after beginning resynthesis, aliquots of the reaction mixtures were removed, extracted with $HCCl_3$, and kept on ice until the experiment was complete. The samples were then digested with *Sph I* and analyzed by electrophoresis through an 8% denaturing acrylamide gel (14).

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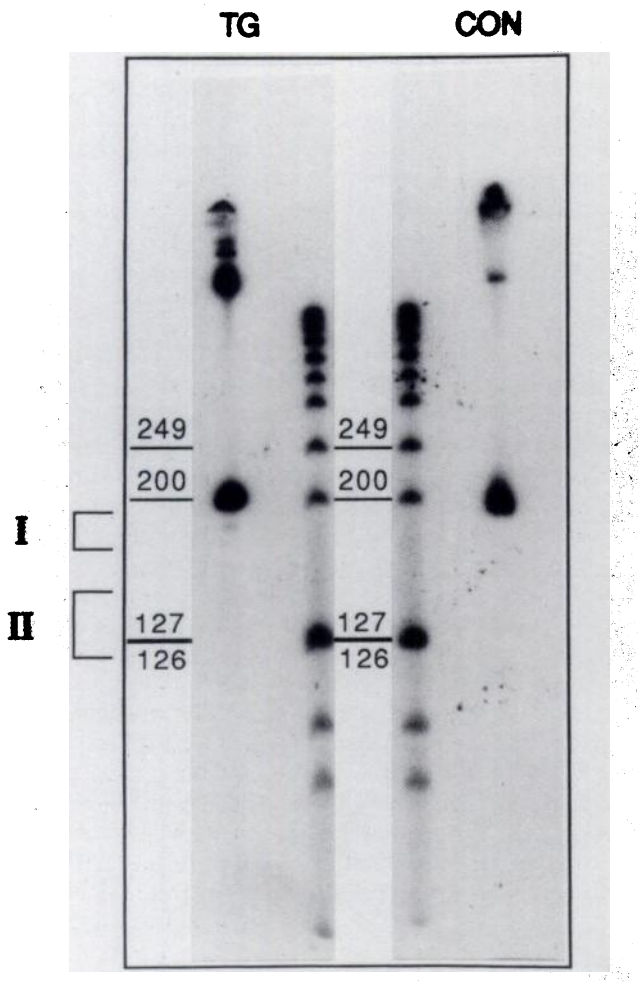

Fig. 8. Verification of complete resynthesis for material used in the binding assay shown in Fig. 10. Resynthesis of Exo III-treated pJLO was performed using 1 mM dATP and dTTP, 100 μM α-^{32}P-dCTP (final specific activity 2 μCi/nmol), and either 1 mM dGTP or 200 μM TdGTP. After incubation for 60 min at 37°, the reaction was stopped by extraction and aliquots were digested with *Sph I* and analyzed as in Fig. 7.

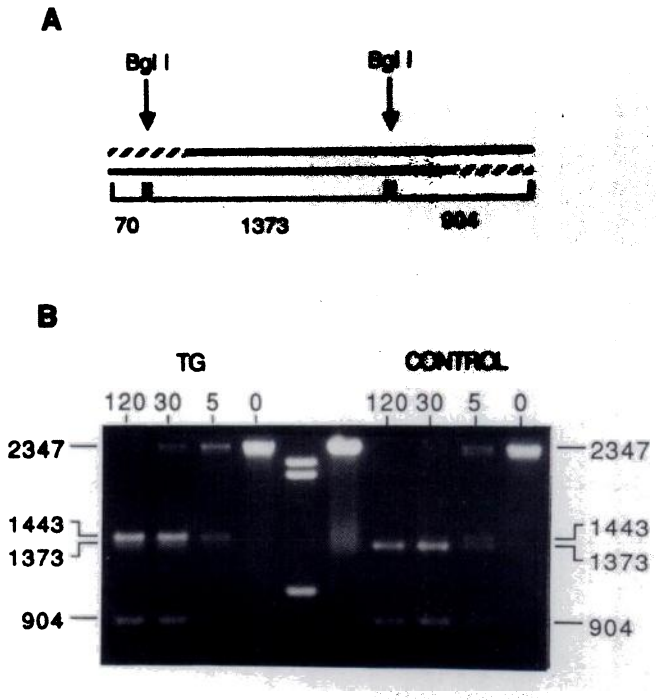

Fig. 9. Inhibition of *Bgl I* digestion by incorporated TG. Limit digestion of *Hind III*-cut pJLO with *Bgl I* gives fragments of 70,904, and 1373 bp as shown in A. A time course of *Bgl I* scission of Exo III-treated pJLO resynthesized with normal or TdGTP-containing nucleotide mixtures was carried out by incubating 4 μg of DNA with 8 units of *Bgl I* (Bethesda Research Laboratories) in 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl at 37°. At the indicated times (min) 0.95-μg aliquots were removed and extracted with $HCCl_3$. The products were analyzed on a 1.5% agarose gel (B).

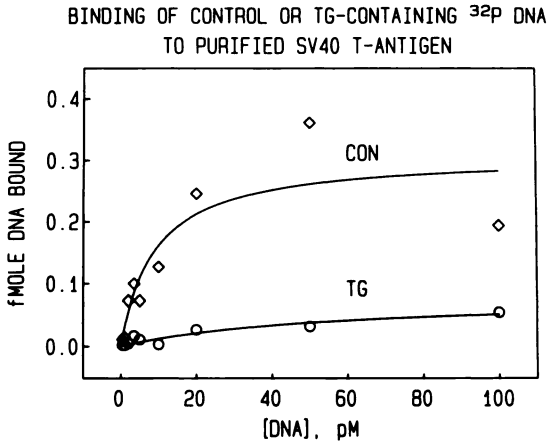

Fig. 10. Binding of ^{32}P-labeled resynthesized pJLO DNA to SV40 T-ag was measured by adsorption to nitrocellulose filters, as described in the text. One of duplicate experiments yielding similar results is shown.

unique *Bgl I* scission site (31). Most of the replication machinery is provided by the host cell, with the exception of one viral gene product, T-ag. Functional T-ag is an absolute requirement for initiation of replication, although initiated molecules can complete replication without competent T-ag (32). It has been shown that T-ag binds specifically to three sequences in the origin region, one of which contains a 27 base pair, G/C rich, perfect palindrome, and it is thought that interaction of T-ag with this sequence may be critical for initiation (33).

One possibility to be considered is that incorporation of TG into the T-ag-binding site disrupts the structural features which are needed for the protein to recognize and bind to this sequence. In this case one might predict that since the TG substitutions are presumably located at random, only the fraction of substituted DNA molecules which happened to contain TG at a critical site (or sites) would be affected. This would explain why only a relatively small portion of the viral molecules was rendered uninitiable. Such an explanation would also account for the ineffectiveness of TG as an antiviral agent (where there are few target regions per viral genome) compared to its effectiveness as a cytotoxic agent (where there are thousands of potential targets, i.e., replication origins, per genome). We therefore proceeded to test directly the effects of TG incorporation on T-ag binding in a cell-free system.

Construction of TG-substituted SV40 origin se-

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quences. The approach chosen for producing analog-containing sequences is a relatively simple one, using commonly available enzymes and requiring only that the 5′-triphosphate of the desired nucleoside be obtained. In the present case this precursor was synthesized from the parent deoxynucleoside by a two-step procedure which has been successfully applied for the synthesis of several other analog triphosphates (15). We were somewhat surprised that resynthesis with the TdGTP-containing mixture was apparently stalled at discrete sites, since it had been previously reported that TdGTP was essentially as good a substrate as dGTP for some DNA polymerases, when using activated calf thymus DNA as a template (25). Townsend and Cheng (34) found that *in vitro* DNA synthesis using ara-CTP or ara-aza-CTP in place of dCTP was substantially impeded when the template sequence required placement of two of these analogs in succession, and it is possible that a similar phenomenon may take place when insertion of two adjacent TG moieties is attempted. Although the stalling out of *in vitro* resynthesis makes construction of substituted sequences more difficult, it is unlikely that this phenomenon has great significance in the intact cell, where dGTP is present along with TdGTP, as indicated by the facility with which cells progress through the first round of replication in the presence of TG (6).

Inhibition of *Bgl I* scission by the presence of incorporated TG. The effects of analog incorporation on restriction endonuclease action have been characterized in a number of different systems and appear to be highly variable, depending upon both the nature of the analog and the particular restriction enzyme under consideration (summarized in Ref. 35). In most cases the analogs studied have been naturally occurring modifications of dCyd or dThd, such as dUrd, 5-hydroxymethyl-dUrd, 5-hydroxymethol-dCyd, or glucosylated 5-hydroxymethyl-dCyd. Non-naturally occurring analogs have rarely been studied in this context, with the exception of BrdUrd. Incorporation of Brd Urd generally causes a slight to moderate decrease in the rate of cleavage by restriction endonucleases, although cutting by Mbo I is enhanced 5-fold by Brd Urd substitution (36). Given the diversity of response in these systems, we had no preconceived expectation as to the effect of TG on *Bgl I* activity. The experiment shown in Fig. 9 demonstrates quite clearly that the presence of incorporated TG in or near the recognition site strongly inhibits *Bgl I* scission. Since the unreconstructed site is cut normally, we conclude that the presence of incorporated TG did not inactivate the enzyme.

Effect of TG incorporation on T-ag binding and potential significance to cytotoxicity. The results from our studies using TG-treated, intact cells infected with SV40 virus suggested to us that the presence of TG incorporated into the replication origin may disrupt T-ag binding to that locus, thereby inhibiting initiation of new rounds of viral DNA replication. Of the three T-ag-binding sites present within the SV40 origin region, the one at which replication begins (site II) has the second highest affinity for T-ag and has not been amenable to characterization by filter binding assays. We therefore elected to substitute both site II and the highest affinity binding domain (site I), which is responsible for autoregulation of T-ag transcription (18). In doing so, we recognize that the disruption of binding we observe is attributable primarily to the interaction of T-ag with site I, and that TG incorporation may not affect these two binding sites in the same way. The main point, however, is that we have shown that the presence of TG incorporated into T-ag recognition sequences has a substantial impact on the binding of that protein.

Although our results are consistent with the hypothesis that inhibition of replication of TG-containing SV40 DNA is due to diminished T-ag binding, the significance of these findings to the cytotoxic mechanism of TG in intact cells is more difficult to assess. Until recently, descriptions of the mechanism responsible for TG cytotoxicity have rarely gone beyond the observation that it is often related to incorporation of the analog into DNA. In the last few years it has been shown that this incorporation is closely associated with disruption of DNA structure as manifested by a characteristic form of macroscopic chromosome disruption (8, 9), by the appearance of single-strand breaks or alkalai-labile sites (30, 37, 38), and by the formation of DNA-protein crosslinks (39), all of which may contribute to loss of viability. Since the structure and integrity of DNA are intimately related to its interactions with proteins in both sequence-specific and sequence-independent modes, it is possible that disturbance of such interactions resulting from TG incorporation, as demonstrated here, may be an integral step in mediating some or all of the effects noted above. For example, the presence of TG within some replication origin sequences in cellular DNA could prevent initiation at those sites, resulting in delayed or incomplete replication of some portion of the genome.

The idea that incorporated analogs can interfere with critical DNA-protein interactions has been addressed previously, primarily using BrdUrd as an example. The presence of incorporated BrdUrd has been shown to alter the affinity of DNA for intercalated drugs (40) and both sequence-specific and non-sequence-specific binding of proteins (41, 42). In addition, BrdUrd treatment can cause a wide variety of cellular responses which most likely result from changes in gene expression (43). However, the effects of most other synthetic analogs (especially those of therapeutic significance) on DNA protein interactions have not been evaluated. It seems likely that analogs will not affect all sequence-specific protein binding interactions in the same way and that it may be possible to define structure-activity relationships for predicting how a particular analog, once incorporated, will change a given protein-DNA interaction. In order for such a scenario to take place we must acquire a deeper understanding of the interactions between both normal and analog-containing DNA sequences, and the proteins which bind to them.

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