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EFFECT OF 1,4-DIHYDROXY-5,8-BIS (2-(2-HYDROXYETHYLAMINO) ETHYL AMINO)-9,10-ANTHRACENEDIONE(DIHYDROXYANTHRAQUINONE) ON THE REPLICATION OF SIMIAN VIRUS 40 CHROMOSOME

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SUMMARY: The effect of dihydroxyanthraquinone on mammalian chromosome structure and replication was investigated using simian virus 40 chromosome as a model system. Viral DNA synthesis in African green monkey kidney cells was approximately 90% inhibited by the drug at 0.4 μM. RNA or protein synthesis was inhibited only 50% under the same conditions. Both single-stranded and double-stranded breakage of viral DNA were found on viral chromosomes isolated from infected cells treated with the drug. Four distinct viral chromosomal templates were found in nuclear extract prepared from cells treated with the drug as determined by the cell-free system for viral DNA synthesis. The results suggested that dihydroxyanthraquinone acts at the level of chromosome replication.

INTRODUCTION

Dihydroxyanthraquinone(DHAQ) is one of the aminoanthraquinone derivatives that has potential anticancer activity(1-3). Its therapeutic value is currently under vigorous clinical studies(Phase II)(4,5). The exact mode of action of DHAQ in mammalian cells is not known. Generally, DHAQ is thought to exhibit anticancer activity because of its ability to intercalate into DNA helics. However, not all intercalating agents have effective anticancer activity(6). Thus, this property alone does not appear to be sufficient to explain the mode of action of anticancer drugs. DNA in eucaryotic cells is complexed with both histone and nonhistone proteins to form a chromosome structure(7). It would be more realistic to investigate the possible mode of action of this drug on DNA structure and replication in a system where these proteins are present.

Abbreviations: EGTA, Ethylenebis (oxyethyenenitrilo)-tetraacetic acid; EDTA, ethylene diamine tetraacetic acid; HEPES, N-2-hydroxyethlpiperazine-N'-2-ethanesulfonic acid; dNTP(s), deoxynucleoside triphosphate(s); rNTP(s), ribonucleoside triphosphate(s); TdR, thymidine.

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Among the DNA tumor viruses, simian virus 40(SV40) and polyoma are simple model systems for studying the mechanism of mammalian chromosomal structure and DNA replication. Viral DNA complexes with histone and nonhistone proteins to form "minichromosome" in the nucleus(8). Approximately 22 nucleosomes are randomly distributed around one molecule of SV40 DNA. Viral DNA replicates discontinuously in a semiconservative fashion. Thus, SV40 chromosome may be considered analogous to an individual eucaryotic replicon(9). In this report, I have used SV40 as a model to study the effect of DHAQ on chromosome replication in vivo and in vitro.

METHODS

Cells and Virus: African green monkey kidney(CV-1) cell line was routinely grown in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum and gentamycin(0.2 μg/ml). The small plaque SV40 strain, Rh911 was routinely propagated in CV-1 cells(11).

Measurement of DNA, RNA and protein synthesis: Cell monolayers(60 x 15 mm) were infected with SV40 at multiplicity of infection(m.o.i.) of 50 for 42 hours at which time the rate of viral DNA synthesis reached a maximum. Infected cells were incubated with different media containing either ^{3}H-thymidine(20μCi/ml), ^{3}H-uridine (100μCi/ml), or ^{3}H-amino acids hydrolysate(100μCi/ml) for the determination of DNA, RNA or protein synthesis, respectively. After one hour incubation at 37°C, cells were lysed with 0.5% of sodium dodecylsulfate(SDS) and the cell lysate was precipitated with cold 1 N HCl. The acid-insoluble radioactivity was determined in a Beckman LS-250 liquid scintillation counter(11). To differentiate the viral and cellular DNA synthesis, infected cells were lysed by the method described by Hirt (10). The acid-insoluble radioactivity of the Hirt supernatant was designated as viral DNA.

Preparation of nuclear extract: At 36 hours after infection, cells were replaced with medium containing ^{3}H-TdR(10μCi/ml) for 10 hours. At 50 hours after infection, five plates of cells were replaced with medium containing DHAQ(3 μM). At 55 hours after infection, cells were lysed by Dounce homogenizer in hypotonic buffer containing 10 mM HEPES, pH 7.8, 5 mM KCl, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.45 mM dithiothreitol, 2 mM ATP, 5 mM phosphoenolpyruvate, 3 μg of pyruvate kinase, 0.3 mM phenylmethylsulfonyl fluoride, 100 M each of dATP, dTTP, dGTP, dCTP, UTP, GTP and CTP and 100 μl of nuclear extract(11). Experiments using (α-^{32}P)dCTP(30-50 Ci/mmole) contained 10 μM dCTP in the reaction mixture. (α-^{32}P)dCTP with a specificity of 30-50 Ci/mmole was routinely prepared by the method of Johnson and Walseth(13).

Agarose gel electrophoresis: After the termination of DNA synthesis in vitro, viral DNA was extracted and separated on a linear 5-20% sucrose gradient as described by Su and DePamphilis(12). Fractions containing SV40 DNA were pooled from the gradient and electrophoresed on a 1.4% agarose slab gel at 25 mA overnight at 4°C (12). At the end of the run, the gel was stained with eithidium bromide(5 μg/ml) for 15 min. and photographed. Autoradiography was carried out at -70°C.

RESULTS

SV40 infection of both permissive and nonpermissive cells stimulate cellular DNA synthesis(14). The presence of 1.6 nM of DHAQ in the medium inhibited appro-

TABLE 1. The effect of DHAQ on DNA, RNA and protein synthesis in SV40 infected cells[a,b]

DHAQ Conc. (M)	% of synthesis			
	DNA		RNA	Protein
	Hirt pellet	Hirt sup.		
Control	100	100	100	100
1.6×10^{-9}	63.2	50.0	-	-
4.0×10^{-8}	24.6	16.8	-	-
4.0×10^{-7}	11.5	13.8	43.4	49.4
8.0×10^{-7}	9.6	13.8	35.7	38.5
1.6×10^{-6}	12.4	9.8	-	-
4.0×10^{-6}	9.2	9.5	45.3	42.6
8.0×10^{-6}	10.2	7.3	42.9	47.6

[a] At 33 hours after infection, cells were labeled with ^{3}H-thymidine, ^{3}H-uridine, or ^{3}H-amino acid hydrolysate for one hour at 37°C. Viral and cellular DNA synthesis was determined as described in the Methods.

[b] Each value was the average of three independent experiments.

ximately 50% of both cellular and viral DNA synthesis(Table 1). Nearly 90% of the inhibition was observed at a DHAQ concentration of 0.4 μM or higher. The inhibition of DNA synthesis was completed within 10-15 min. Approximately 10% of residual DNA synthesis was apparently resistant to the effect of DHAQ. It is interest to note that the normal cell monolayer remained intact and viable, judged by vital staining, even after DNA synthesis was inhibited for 18 hours. In virus-infected cells, viral DNA synthesis resumed after the removal of the drug from the medium and eventually led to the lysis of cells.

Both RNA and protein synthesis was inhibited 50% in the presence of DHAQ (0.8-8 μM)(Table 1), when DNA synthesis was 90% inhibited. Thus, it is unlikely that the inhibition of SV40 DNA synthesis was merely a secondary effect of the inhibition of RNA and protein synthesis. Judging from the similar total radio-activity(acid-insoluble and acid-soluble) found in cells treated with or without DHAQ, the uptake of thymidine was not affected by DHAQ in the medium.

Prelabeled ^{3}H viral chromosomes isolated from cells treated with DHAQ for 5 hours, showed the same sedimentation value(70S) on sucrose gradient as that of

TABLE 2. The effect of DHAQ on the distribution of SV40 DNA synthesized *in vivo*[a] and *in vitro*[b]

DNA synthesis	cell treatment	% of different forms of SV40 DNA[c]			
		SV40(RI)	SV40(II)	SV40(III)	SV40(I)
In vivo:					
	without DHAQ	5.2	9.2	8.5	77.0
	with DHAQ	7.8	18.3	17.4	59.0
In vitro:					
	without DHAQ	34.8	21.0	9.8	34.2
	with DHAQ	20.9	32.2	15.5	31.2

[a]The nuclear extract containing ^{3}H viral chromosomes was analyzed on a 5-30% linear sucrose gradient(11). The viral chromosomes were then identified and pooled for the analysis of viral DNA in a 1.4% agarose gel. Different forms of viral DNA were stained with ethidium bromide, sliced, and solubilized as described in the Methods. The radioactivity was measured in scintillation fluid.

[b]Viral DNA synthesized in nuclear extract in the presence of(α-^{32}P)dCTP was extracted and analyzed on a 1.4% agarose gel. The ^{32}P viral DNA was sliced from the gel, solubilized, and determined for radioactivity.

[c]Viral mature(SV40(I)) DNA is a supercoiled covalently closed DNA. A breakage occurring on either strand of SV40(I)DNA results in a relaxed, circular (SV40 (II))DNA. Viral linear (SV40(III)) DNA is generated by cleaving the phosphodiester bonds on both DNA strands.

the normal viral chromosome(12). However, only 59% of the supercoiled SV40 DNA was found in viral chromosomes isolated from DHAQ-treated cells as compared to the 77% of supercoiled DNA generally found in normal viral chromosomes(Table 2). Different configurations of SV40 DNA molecules which can be easily be resolved on agarose gel provide an unambiguous approach to measure the DHAQ induced strand breakage. Approximately two fold increase of the amount of relaxed, circular SV40(II)DNA and linear SV40(III)DNA was found in SV40 chromosomes obtained from DHAQ-treated cells(Table 2).

Intercalating agents generally share the properties of reversible binding to DNA(6, 15). Therefore, the template activities can be measured after the removal of DHAQ. DNA synthetic activity was found higher in the nuclear extract obtained from cells treated with DHAQ than that from control cells(Table 3). Relatively, similar amount of the supercoiled SV40 DNA were synthesized by nucear extract treated in the absence and presence of DHAQ, yet, approximately 50% more SV40(II)

TABLE 3. Viral DNA synthesis in nuclear extract of SV40 infected cells with and without DHAQ[a]

Cell treatment	Aphidicolin[b] (10^{-4}M)	Prelabeled ^{3}H SV40 DNA (x 10^{-3}cpm)	DNA synthesized ^{32}P SV40 DNA (x 10^{-3}cpm)	$^{32}P/^{3}H$
without DHAQ	-	6.1	17	2.97
	+	5.5	6.1	1.10
with DHAQ	-	4.6	25	5.43
	+	3.3	5.6	1.70

[a]At 55 hours post-infection, nuclear extracts were prepared from infected cells treated with and without DHAQ as described in the Methods. Viral DNA synthesis in nuclear extract was assayed in the standard reaction mixture containing (α-^{32}P)dCTP for 60 min.

[b]Approximately 85% of cellular and viral DNA synthesized in vivo was inhibited.

and SV40(III)DNA was synthesized by nuclear extract in DHAQ-treated cells(Table 2). Aphidicolin, a specific inhibitor of α DNA polymerase(16), inhibited approximately 70% of DNA synthetic activity in nuclear extract(Table 3). The result indicated that α DNA polymerase was the major enzyme responsible for DNA synthesis in cells treated with and without DHAQ. The residual amount of DNA synthesized in the presence of aphidicolin was apparently not associated with replicating templates since no ^{32}P DNA was seen on gel(Figure 1, Lane B, C).

DISCUSSION

The results obtained in this study show that both cellular and viral chromosome replication are inhibited to the same extent in cells treated with low concentration of DHAQ(Table 1). Viral chromosomes isolated either from DHAQ-treated cells or incubated directly with DHAQ at 2-10 μM showed no detectable change in the sedimentation on sucrose gradient. But, aggregation of viral chromosomes occurred when higher concentrations of DHAQ(>20 μM) was present(data not shown).

Like other intercalating agents, such as adriamycin and actinomycin D(17, 18), DHAQ induced both single and double strand breaks on SV40 chromosomal DNA in cells (Table 2). The exact mechanism involved is currently not known. It is possible

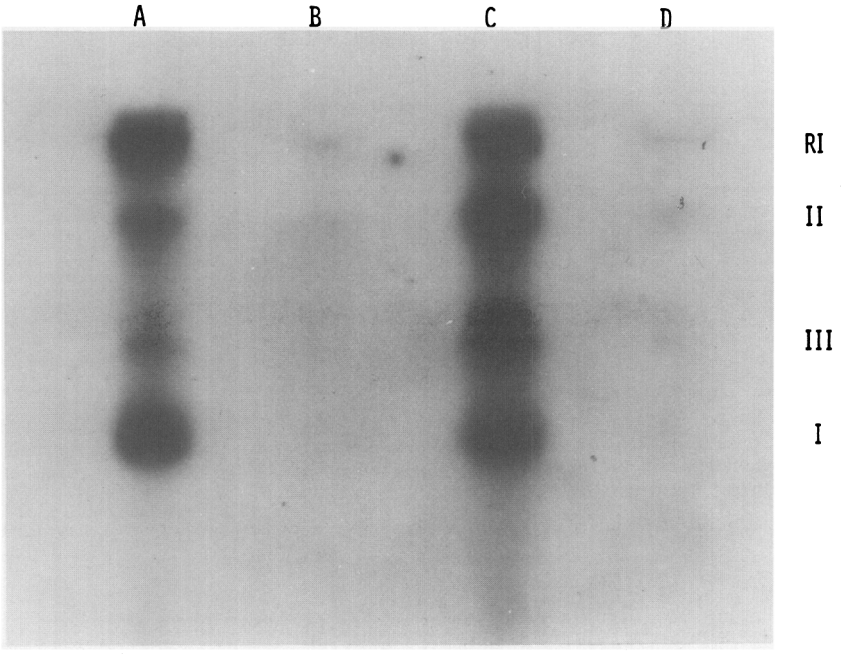

Figure 1. Gel electrophoresis of SV40 synthesized in vitro.
Nuclear extract obtained from infected cells treated with(C,D) and without (A,B) DHAQ was assayed for viral DNA synthesis in the presence of (α-^{32}P) dCTP. Aphidicolin was included in nuclear extract(B,D). The autoradiography was carried out at -70°C. RI, SV40(RI)DNA; II, SV40(II)DNA; III, SV40(III)DNA; I, SV40(I)DNA.

that the interaction of DHAQ with viral DNA perturbs the nucleosome arrangement and causes the excision of DNA molecule by nuclease as suggested(17) or the strand scission may be generated by the free radicals formed from the metabolites of DHAQ as demonstrated in cells treated with adriamycin(19). If DHAQ acts at a specific location on the SV40 genome, this site can be identified by the use of restrictive endonucleases since the entire SV40 DNA sequence is known(14). This is a distinct advantage of SV40 chromosome compared to cellular chromosome where the exact location of DNA breakage is difficult to identify because of the complexity of the genome size.

It is likely that ^{32}P SV40(II) and ^{32}P SV40(III) DNA synthesized in vitro were the result of repair synthesis on the damaged templates since viral DNA strand breaks were detected(Table 2). But these viral DNA might also be the result of the accumulation of viral templates at intermediate stage of replication

(9), because addition of DHAQ to cells caused an immediate cessation of both cellular and viral DNA synthesis.

DHAQ has been shown to be preferentially accumulated in nucleoli and to bind to chromatin in Chinese hamster ovary cells(20, 21). Prolonged incubation of cells with DHAQ at the concentrations that inhibited DNA synthesis showed no cell lysis implying that the membrane was not damaged extensively. The results obtained from this study and others(18, 21) support the idea that the primary target of DHAQ is at the chromosome level. SV40 chromosome should provide a unique experimental tool to investigate the molecular mechanism of DHAQ on chromosome replication.

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