Differential effect of DNA supercoiling on transcription of adenovirus genes in vitro

Subhasis Banerjee*† and David J. Spector²

1 Department of Pathology, The Royal Veterinary College, University of London, London NW1 0TU, U.K. and 2 Department of Microbiology and Immunology, The Pennsylvania State University, College of Medicine, Hershey, Pennsylvania 17033, U.S.A.

We examined the effect of DNA template topology on the transcription of immediate early (E 1 a), early (E 1 b) and late (pIX) adenovirus genes in vitro. Transcription in whole cell extracts was measured by quantitative hybridization to end-labelled DNA and protection of hybrids from S1 nuclease digestion. Two- to fourfold more E 1 a RNA was synthesized from supercoiled, compared to linear, DNA templates. Similarly, transcription of the E 1 b gene was stimulated three- to sevenfold when the template was supercoiled. In contrast, RNA synthesis from the late pIX gene was found to be independent of DNA topology. These results show that DNA topology affects transcription in a promoter-specific manner.

Introduction

Many components contribute to the efficiency of RNA polymerase II (pol II)-mediated transcription. Of these, the best documented are the actions of auxiliary transcription factors (McKnight & Tjian, 1986) and the conformation of the genome promoter region (Drlica, 1984; Reeves, 1984). However, DNA topology, which often affects conformation (Cantor et al., 1988), also has been implicated in modulating RNA synthesis in vivo and in vitro (Smith, 1981; Drlica, 1984; DiMauro et al., 1985; Weintraub et al., 1986; Cantor et al., 1988; Huang & Roeder, 1988; Hirose & Suzuki, 1988). Bacterial RNA polymerase recognizes bacteriophage and plasmid DNA more efficiently when the templates are supercoiled (Drlica, 1984), whereas specific interactions between yeast RNA pol II and cloned yeast genes also depend upon DNA supercoiling (DiMauro et al., 1985). In other cases, DNA supercoiling appears to have no effect on transcription (Matsui, 1987; Hirose & Suzuki, 1988) or, rarely, increased unwinding of DNA inhibits RNA synthesis (Brahms et al., 1985).

DNA-containing animal viruses, especially adenovirus and simian virus 40 (SV40), are useful model systems to study the molecular mechanisms of gene expression. One interesting feature of the life cycle of these viruses is the precise temporal regulation of gene expression during productive infection (Tooze, 1981). For example, the adenovirus immediate early (E 1 a), early (E 1 b) and late (pIX) genes belong to different temporal classes. The E 1 a gene is expressed soon after infection, and one of its products activates transcription of E 1 b (Berk et al., 1979; Jones & Shenk, 1979; Ricciardi et al., 1981). The pIX gene encodes a capsid protein and is expressed late (Crossland & Raskas, 1983). A number of investigators have attempted to elucidate the mechanisms controlling these transcriptional switches, with inconsistent results (Crossland & Raskas, 1983; Matsui et al., 1986; Venkatesh & Chinnadurai, 1987; Vales & Darnell, 1989). For instance, earlier work suggested that viral DNA replication was a prerequisite for activation of the pIX gene. A recent report suggests that, provided that the contiguous E 1 b and pIX genes are physically separated, DNA replication is not necessary to induce pIX transcription (Vales & Darnell, 1989).

The adenovirus genome is a double-stranded linear DNA that can assume a circular configuration via the self-association of a protein bound to the 5' end of each strand (Robinson et al., 1973). The viral nucleoprotein might assume higher order structures in the infected cell or in the virion (Corden et al., 1976; Tate & Philipson, 1979). The formation of such structures could involve distinct topological changes during infection and such changes might affect transcription. Indeed, a recent report shows that adenovirus DNA is folded into multiple loops with torsional constraints that can be relieved by topoisomerase I (Wong & Hsu, 1989). Moreover, genomic organization of the penton-less virion can undergo structural transitions during infection.
In order to determine whether promoter topology influences the transcription of temporally regulated genes in vitro, we compared RNA synthesis from E1a, E1b and pIX promoters in deproteinized linear and supercoiled templates. We show that these three promoters fall into two classes based on quantitative analysis of RNA transcribed from linear and supercoiled templates.

**Methods**

*Virus, viral DNA and plasmids.* The recombinant DNA pXC1 is a pBR322-derived plasmid containing the left 16% of the adenovirus 5 genome (XhoI site). The plasmid was grown in mass culture and purified as described by Birnboim & Doly (1979) and Parks et al. (1988).

*DNA templates for transcription.* To prepare linear templates, pXC1 DNA was cleaved with appropriate restriction enzymes (New England Biolabs) according to conditions specified by the supplier. DNA was purified by phenol/chloroform extraction followed by ethanol precipitation. Supercoiled template was purified by taking advantage of the ability of supercoiled DNA to renature rapidly following denaturation. In these conditions, strands derived from relaxed and nicked forms remain single-stranded and can be bound selectively to nitrocellulose membranes. Approximately 50 μg of DNA in 10 mM-Tris·HCl pH 7.5 and 1 mM-EDTA was denatured by adjusting to 0·1 M-NaOH. After mixing for 5 min at room temperature, the solution was neutralized by adding Tris·HCl pH 7·5 and HCl, each to 0·1 M. The salt concentration was adjusted to 0·4 M with NaCl, and the slowly annealing fraction (single-stranded) was removed by slowly filtering the solution through two nitrocellulose membranes (BA-85, Schleicher & Schuell). The supercoiled DNA was isolated in the filtrate.

Covalently closed, relaxed circular DNA was prepared by treatment of 100 μg/ml supercoiled DNA with 200 units/ml of topoisomerase I (Bethesda Research Laboratories) for 30 min at 37 °C. Nicked circular DNA was prepared by limited DNase I digestion (Wang, 1974). Supercoiled DNA at a concentration of 166 μg/ml was treated with 100 ng/ml DNase I for 10 min at 0 °C. Under these conditions, a mixture of approximately equal amounts of supercoiled and nicked DNA was obtained. The nicked DNA was isolated by electrophoresis from agarose gels. Catenanes (see Results) were isolated from the origin of low melting point agarose gels (FMC) by melting the agarose to elute the DNA.

The different forms of DNA present in the preparations were examined by agarose gel electrophoresis. Supercoiled DNA preparations had greater than 90% form I DNA, as visualized in ethidium bromide-stained gels. Other DNA preparations did not contain any detectable contaminating forms. When the purified nicked DNA preparations were run on denaturing agarose gels, about equal amounts of unit length circular and linear single-stranded molecules were observed, with no evidence of degradation which would indicate multiple nicks in the DNA strands. This result is consistent with previous observations that nicked DNA prepared by this procedure consists primarily of molecules with one single-strand break (Wang, 1974). Both the nicked and catenated DNAs, which were isolated from agarose gels, could be cut with restriction endonucleases. The DNA concentrations of all templates were determined spectrophotometrically by measuring absorbance at 260 nm.

In vitro transcription. The preparation of whole cell extracts for in vitro transcription and the appropriate reaction conditions for RNA synthesis have been described by Parks et al. (1988). Transcription was carried out in 25 μl reaction mixtures containing 12 mM-HEPES pH 7·9, 60 mM-KCl, 7·5 mM-MgCl₂, 0·6 mM-EDTA, 1·2 mM-DTT, 12% glycerol, 500 μM each of ATP, CTP, GTP and UTP, 1 mM-creatine phosphate, 3 to 15 mg/ml of extract protein and DNA. No non-specific carrier DNA was added. The extract was preincubated routinely for 15 min at 30 °C before addition of the rNTPs mixture (including creatine phosphate) and the template DNA to initiate RNA synthesis.

Nucleic acid was purified by treatment with 7 M-urea, SDS and phenol extraction (Holmes & Bonner, 1973), and the template DNA was degraded by treatment with DNase I. The RNA products synthesized were analysed by hybridization in solution to 5'-32P end-labelled DNA probes (Berk & Sharp, 1978; Weaver & Weissman, 1979; Parks et al., 1988) and nuclease-resistant labelled DNAs were resolved by electrophoresis at 40 °C in 1·5 mm, 4 to 6% denaturing polyacrylamide vertical slab gels. RNA synthesis was quantified by densiometric scanning of specific bands on preflashed autoradiograms (Laskey & Mills, 1977). The pre-exposing of XR-5 film was done by placing the film over a yellow paper and exposing a single flash unit with an orange filter (170 BC computer soft light reflect). Flashing at a distance of 70 cm gave an average increase in background of 0·12 to 0·15 OD units at 540 nm compared to unexposed films. A reconstruction analysis employing serial dilutions of radioactively labelled transcripts was performed to ensure a linear relationship between the radioactivity of the sample and absorbance of the film image after preflashing. The optical density of specific bands was measured at 540 nm using a Beckman DU-8 spectrophotometer with a gel scanning accessory. The reliability of this method of quantification was further verified by direct scintillation counting of the radioactive bands cut out of the wet or dry gels. However, the data presented here represent the relative number of transcripts (not absolute numbers) per template based on the fact that, because probes were end-labelled, the SI protection signal is a direct measurement of relative transcript number.

**Analysis of the fate of template DNA in transcription extracts.** Nucleic acid was prepared and incubated as for transcription reactions and then isolated from assay mixtures by SDS/protease K treatment and phenol extraction (Mertz, 1982). After degradation of RNA with RNases A and T1, the DNA was analysed by agarose gel electrophoresis.

**Electron microscopy.** Catenated DNA isolated from transcription reactions was purified further by electrophoresis in low melting point agarose (FMC) and elution from the gel. For microscopy, the DNA (0·1 μg/ml) was spread in 40% formamide and 0·1 mg/ml cytochrome c. The protein/DNA mixed film was transferred to grids covered with Parlodion film, subsequently stained with uranyl acetate and shadowed with platinum. DNA was visualized in a Philips model 201 transmission electron microscope.

**Results**

*RNA synthesis in vitro, quantitative hybridization and SI nuclease digestion.*

Plasmid pXC1 contains the left 16% of adenovirus 5 DNA (Fig. 1a). These sequences include transcriptional control regions for the three viral genes, E1a, E1b and pIX. Fig. 1(b) depicts the predicted lengths of S' end-labelled DNA fragments protected from SI nuclease digestion after hybridization to those RNAs synthesized in vitro. We first asked whether template topology could affect E1a and E1b transcription. Initially transcription
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Fig. 1. (a) Structure of recombinant plasmid pXC1. The adenovirus 5 DNA is shown as a solid bar. The pBR322 sequences, with restriction site positions and nucleotide numbers in parentheses, are shown as a thin line. The location of the first base pair in some landmark restriction enzyme sites and the size of the plasmid are indicated. (b) Strategy for hybridization analysis of adenovirus E1a, E1b and pIX transcripts. The solid bar represents the viral DNA sequences in pXC1, whereas the thin line indicates pBR322 sequences. Also shown are the location of transcription start sites (Baker & Ziff, 1981; Petterson & Mathews, 1977): the arrows show the direction of transcription. The restriction enzyme cleavage sites used to generate probes are shown above the line. The asterisks represent the positions of 5′ end-label of the DNA strand complementary to the RNA. The thick solid lines below show the predicted sizes of the labelled DNA fragments protected from S1 nuclease digestion after hybridization to the RNAs (Fire et al., 1981). The KpnI and BstEII probes were the complete linearized pXC1. The purified BstEII fragment used as a probe extended through pBR322 sequences to another site that begins at adenovirus nucleotide 5186. The purified SacI fragment probe extended to another site that begins at adenovirus nucleotide 354.

was measured for linear and supercoiled templates at a concentration of 40 μg/ml (Fig. 2). Prominent bands were obtained of the sizes corresponding to authentic E1a (1550 nucleotides) and E1b (350 nucleotides) transcripts. The other products observed probably represented incomplete protection of E1a RNAs, as their mobility changed predictably with the size of the probes used to detect the E1a and E1b products (data not shown). These reaction products were synthesized by RNA pol III, as indicated by their sensitivity to 0.5 μg/ml of α-amanitin and were only seen when template was added (data not shown). Another template-dependent band was seen in some experiments and its appearance was α-amanitin-resistant (data not shown). This band

was observed only with some linear templates and probes (see Fig. 3). Since its mobility shifted when the restriction site used to generate linear templates was altered, it probably corresponded to residual template DNA protected by the probe.

DNA supercoiling stimulates transcription from E1a and E1b promoters

Quantification of RNA synthesized (Fig. 2) showed that about threefold more E1a transcripts and about sixfold more E1b transcripts were made from supercoiled templates than from linear ones. For this and subsequent experiments carried out at different template concentrations, transcriptional efficiency was expressed as transcripts made per template copy per hour, a measurement of specific template activity (see Methods). The optimal DNA concentration for transcription was defined as that at which the specific template activity was maximal. This concentration did not always coincide with that at which maximum RNA synthesis was obtained. In this experiment the DNA concentration (40 μg/ml) was optimal for transcription of pXC1 DNA with the extract used.

We carried out many similar experiments with different extracts prepared from KB and HeLa cells. Although the relative efficiency of transcription of each gene varied from extract to extract, two- to fourfold more E1a RNA and three- to sevenfold more E1b RNA was made from supercoiled templates.
We considered the possibility that RNA made from *KpnI*-generated linear template might not completely protect a probe DNA labelled at the same *KpnI* site. Such a situation might have resulted in underestimation of the amount of RNA made from linear templates in the experiments shown in Fig. 2. Therefore, RNA was synthesized from pXC1 cut with *EcoRI* which cleaves the DNA in the pBR322 sequence, and assayed with *KpnI*- or *BsrEII*-generated probes (see Fig. 1b). The same increase in E1b RNA synthesis from supercoiled templates was observed (see Fig. 3). Linear templates were also generated by cleavage with *BglII* (which cleaves at nucleotide 3332 in the adenovirus sequence of *pXC1*) or with *BamHI*, both of which cut pBR322 (Fig. 1). There was no significant difference in the amounts of E1b RNA made from these templates compared with the other linear templates (data not shown).

The amounts of E1a and E1b RNA made from supercoiled and linear pXC1 were determined as a function of DNA concentration (Fig. 3a). For the hybridization assay in this experiment, *BsrEII* was used to make 5′ end-labelled probes (see Fig. 1b), and *EcoRI*-generated linear molecules were used as templates. Residual amounts of linear template were protected by the probe and produced a band migrating ahead of that due to E1a RNAs. The results indicate that at least twofold more E1a RNA and about fivefold more E1b RNA (Fig. 3b) were synthesized from supercoiled templates at every DNA concentration.

Standard transcription assays were carried out for 60 min. To determine whether the increased amount of RNA made from supercoiled templates represented an increase in the rate of RNA synthesis or an increased stability of the products, rates of E1b RNA accumulation were measured directly. These results (Fig. 4) indicate that RNA made from either template accumulated roughly in a linear fashion for about 60 min. Furthermore, E1b RNA made from the supercoiled form accumulated at a rate fivefold faster than that made from the linear DNA. We conclude that the differences observed in RNA accumulation in the 60 min assay reflect true differences in the rate of synthesis of transcripts.

**Transcription from the pIX promoter is unaffected by DNA topology**

We next examined transcription from the pIX promoter in linear and supercoiled pXC1 DNAs. The pIX RNA...
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(a) L Sc L Sc
-Elb
-plX
1 2 3 4

(b) Elb plX
L Sc L Sc
1 2 3 4

Fig. 5. Elb and pIX RNA synthesis in vitro from linear (L) and supercoiled (Sc) pXC1 DNA. (a) Transcription reactions contained 40 μg/ml linear (KpnI-generated) or supercoiled template. Using separate transcription reactions, Elb RNA synthesis was measured by hybridization to a KpnI-generated probe (lanes 1 and 2), whereas pIX RNA synthesis was assayed by hybridization to a SacII-generated probe (lanes 3 and 4). Hybridization was for 12 h at 55 °C. The size markers were labelled HaeII fragments of φX174 DNA. (b) Sc or L (EcoRI-generated) pXC1 DNA added at 20 μg/ml was transcribed. The reaction mixture was divided in half and Elb and pIX RNA synthesis was measured as in (a).

was detected using a probe, prepared by SacII digestion of pXC1, which produced a 245 nucleotide protection product (Fig. 1b). We compared Elb and pIX RNA which was synthesized from 40 μg/ml pXC1 DNA, the optimum for the extract used in this experiment. Elb and pIX RNAs were assayed in separate hybridization reactions. The data (Fig. 5a) show the relevant section of an autoradiogram indicating that a band of the size expected for pIX transcripts was obtained using both linear and supercoiled pXC1 templates. However, in contrast to the increased amount of Elb RNA made from the supercoiled DNA, the amount of pIX RNA made from the supercoiled DNA was actually slightly less than that made from linear DNA.

To compare more directly Elb and pIX mRNA synthesis from the two template structures, the products of a single reaction mixture were divided in half and probed in separate hybridization reactions for Elb and pIX RNAs. Because of the variability in extracts that was noted above, multiple experiments were carried out with different extracts. A representative result from one of the experiments is shown in Fig. 5(b). The same amount of pIX RNA was made from either template structure, whereas 4.5-fold more Elb transcription was obtained with supercoiled DNA than with linear DNA. In other experiments, we measured Elb and pIX RNA synthesis from separate templates that contained only a single transcription start site; the results of varying the template structure were identical to those obtained with pXC1 (data not shown).

These results show that pIX transcription from pXC1 DNA is relatively unaffected by the initial structure of the template. Furthermore, the pIX gene transcription provided an internal control for the specificity of the observation of the increased amounts of Ela and Elb RNA synthesis from supercoiled pXC1. We conclude that the increase in Elb RNA synthesis from supercoiled DNA is promoter-specific, and is not a general property of transcription from the pXC1 plasmid.

Supercoiled DNA is modified in the extracts

In whole cell extracts, supercoiled DNA is usually relaxed, nicked, catenated or partly linearized in the presence of cofactors (Sergeant et al., 1984). We examined the structural changes of supercoiled pXC1 DNA under transcription conditions in the presence or absence of ATP and Mg2+. Supercoiled DNA was incubated in standard transcription conditions, except that reactions were incubated at 0 or 30 °C in the presence of Mg2+ alone, or both Mg2+ and ATP. The data (Fig. 6) show that, at either temperature, supercoiled DNA incubated in the presence of Mg2+ alone was mostly relaxed with some topoisomers that were partly supercoiled. Similar results were obtained at 0 °C in the presence of both cofactors suggesting that the relaxation of supercoiled DNA was mediated by topoisomerase I. However, at 30 °C, the inclusion of both cofactors resulted in extensive catenation of DNA with few detectable topoisomers entering the gel. The catenation reaction was inhibited completely by 20 mM-EDTA. These results are consistent with the type II topoisomerase-mediated catenation reaction which requires ATP hydrolysis (Wang, 1974). As noted by others (Waldeck et al., 1983), we observed some superhelical turns in electron micrographs of the purified SV40 and pXC1 catenanes (Fig. 6c and d). Examination of the fate of linear DNA upon incubation under different conditions revealed that most of the DNA remained linear, although some slowly migrating forms, probably concatemers, were produced (data not shown).

Superhelicity of DNA is necessary for maximum Elb RNA synthesis

The results described above prompted us to investigate whether the increased Elb transcription from super-
Fig. 6. Topological changes of supercoiled pXC1 in whole cell extracts in the presence or absence of ATP hydrolysis. Supercoiled pXC1 DNA was incubated in the presence (+) or absence (−) of 0.5 mM-ATP at 0 or 30 °C (a), or in the presence of ATP and the indicated concentration of EDTA (mM) at 30 °C (b). DNA purified from the agarose gel mixtures was analysed by agarose gel electrophoresis. M, markers; Ori, origin of migration; Rel, relaxed; Sc, supercoiled. (c and d) Electron micrograph of purified SV40 and pXC1 catenanes respectively. Bar marker represents 100 nm.

(a) Linear Relaxed Nicked

Catenanes Supercoiled

(c) Transcription of other forms of pXC1 DNA in vitro. (a) The transcription reactions contained the indicated concentration of the appropriate form of pXC1 DNA (µg/ml). Linear DNAs were generated by EcoRI digestion. Elb RNA was measured as described in the legend of Fig. 5. (b) Quantitative expression of data in (a). The autoradiogram was scanned to obtain the data. ○, Supercoiled; ■, relaxed; □, nicked; △, catenanes; ○, linear.

Discussion

In this report, we show that maximal in vitro RNA synthesis from adenovirus Ela and Elb promoters, but not the pIX promoter, requires DNA supercoiling. Our experiment was controlled internally by having the three promoters on the same template DNA. This effectively ruled out the possibility that non-specific template structural properties affected specific transcription.

The differential response of the promoters to DNA supercoiling occurred despite the fact that supercoiled DNA added to the extract under transcription conditions was relaxed and catenated during incubation. Interestingly, a similar phenomenon was observed after DNA-mediated gene transfer into cultured cells. Despite extensive nicking and linearization of transfected DNA a dramatic enhancement of transcription occurred when the initial template topology of SV40 early promoter-containing DNA was supercoiled (Weintraub et al. 1982).
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1986). In cell-free systems, Hirose & Suzuki (1988), using partially purified silk gland transcription extracts, have correlated DNA supercoiling with transcription of the fibrion gene. Increased RNA synthesis was associated with activities in phosphocellulose column fractions which introduced a few superhelical turns and knots into DNA. We were unable to pursue the question of whether catenation of supercoiled DNA was a necessary condition for increased template activity. Following established purification schemes (Dypan & Tjian, 1983), our repeated attempts to separate topoisomerases from transcription activities in HeLa cell extracts were unsuccessful. Moreover, attempts to inhibit catenation of supercoiled DNA in the reaction using VM 26, a topoisomerase II inhibitor (Chen et al., 1984), resulted in extensive linearization of the DNA (S. Banerjee, unpublished results).

Increased RNA synthesis from supercoiled templates might be due either to more effective and stable promoter–transcription factor interactions which can enhance the rate of initiation via open complex formation (McClure, 1985), or to an increase in the rate of RNA chain elongation. The former possibility is more likely since topological constraints on naked DNA energetically favour protein–DNA interactions (Wang, 1974). In HeLa cell extracts, supercoiled SV40 DNA–histone interactions were more efficient than those with covalently closed relaxed circles (Banerjee & Cantor, 1990).

Maximal Ela and Elb RNA synthesis from supercoiled DNA, despite subsequent topological changes, may be explained if initial superhelicity accelerated the formation of active and stable transcription complexes (Davidson et al., 1983), but was not required for maintenance of continued RNA synthesis (Wong & Calame, 1986). We have observed more efficient Elb transcription from supercoiled templates than linear DNA if sarkosyl was added to inhibit reinitiation shortly after the start of reactions (D. J. Spector, unpublished data). This result is consistent with a model whereby supercoiling promotes increased template utilization in the first few minutes of the reaction. Such a model also may account for increased RNA synthesis from non-supercoiled circular templates. On the other hand, RNA polymerase transit around a circular template may produce more efficient reinitiation.

Unlike that of Ela and Elb, pIX transcription was unaffected by DNA topology. The possibility that promoter competition was responsible for this result was ruled out by experiments with the Elb and pIX genes on separate plasmids (data not shown). A similar lack of topological requirement for transcription was observed in chromatin templates assembled in Xenopus extracts (Matsui, 1987). These findings suggest that the promoter structure of the pIX gene is different from those of the Ela and Elb genes. Since the actual number of templates used for transcription is a small fraction, and since we do not know whether the different promoters are utilized on the same or different templates, the question of promoter occlusion can not be addressed directly. However, our results suggest that promoter occlusion does not participate in this in vitro phenomenon since similar results were obtained with the promoters on the same or different templates. Also there are plasmid promoters that may be used; so any occlusion could not be attributed to the adenovirus promoters for certain. There is probably no simple correlation between early and late transcription classes in vitro and sensitivity of transcription in vitro to DNA topology. Transcription from the late adenovirus promoter ElIa late is increased in nuclear extracts when the initial template structure is supercoiled, whereas early ElIa promoter is unaffected (Huang & Roeder, 1988). In the case of ElIa late, covalently closed, relaxed DNA is an equally good transcription substrate as supercoiled; genetic analysis indicates that particular sequence elements may mediate the structural effect. Whether specific components of Ela and Elb promoters are involved in the response to template structure in vitro remains to be determined.

Finally, the topological effect on transcription of adenovirus promoters described here might be relevant to the virus life cycle. The three genes we tested reside within the first loop (2 to 14 map units) of twelve intertwined domains that are supercoiled in virion genomes (Wong & Hsu, 1989). DNA supercoiling induces S1 nuclease-sensitive secondary structures within the control elements of ß-globin genes (Schon et al., 1983) and SV40 DNA (Iacono-Connors & Kowalski, 1986). If such structures are maintained in transcription complexes early after infection, they may facilitate Ela and Elb promoter recognition. Both linear and circular DNA can be assembled into chromatin with physiologically spaced nucleosomes in HeLa cell extracts (Banerjee & Cantor, 1990; Banerjee et al., 1991). It would be interesting to examine whether such promoter-specific effects on RNA synthesis could be established from chromatin templates.

We thank Linda Kudler and Richard Hyman for help with electron microscopy of catenated DNA, Timothy Grierson and Alan Smallwood for artwork and photography, Frank Flamini and C. F. Colle III for technical assistance, Melissa Hill and Alan Smallwood for editorial assistance and William Hendrickson, Samson Jacob and Hamish Young for helpful comments on the manuscript. We also thank the National Cancer Institute (D. J. S.) and SmithKline Foundation (S. B.) for support.

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(Received 23 March 1992; Accepted 11 June 1992)