DNA gyrase stimulates transcription

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ABSTRACT

The nuclear DNA of HeLa cells can now be isolated unbroken and super-coiled. Using DNA gyrase and the untwisting enzyme, we have prepared an allomorphic series of templates derived from this nuclear DNA, and also from the circular DNA of the bacterial virus, PM2. We have then transcribed these templates using 2 different RNA polymerases - from wheat germ and Escherichia coli. Relaxed DNA is transcribed slowly by both polymerases. Supertwisting the naturally-supercoiled templates with gyrase slightly inhibits transcription by the bacterial polymerase but stimulates dramatically transcription by RNA polymerase II from wheat germ.

INTRODUCTION

It is a surprising fact that broken and denatured DNA is a much better template for the eukaryotic polymerases that transcribe unique sequences than is the more 'native' duplex DNA. For example, RNA polymerase II from wheat germ transcribes single-stranded DNA at twenty times the rate of double-helical DNA[1]. Most attempts to improve transcription have been directed towards isolating factors which might stimulate the polymerase like the σ protein of Escherichia coli (for example, see refs 2 and 3). We have examined an alternative possibility - namely that poor transcription results not only from deficiencies in the polymerase but also from those in the template.

The template surface of DNA is buried within the double helix and must be exposed during transcription by unpairing bases. Just such an unpairing is induced by supercoiling the DNA as a torus in a sense opposite to that of the double helix[4]. As a result supercoiled duplexes are cleaved by single-strand specific nucleases[5] and bind single-strand specific chemicals[6]. Supercoils can only be maintained in intact molecules and the very long and fragile eukaryotic DNA is invariably broken when it is purified using conventional procedures. Recently we have devised techniques for isolating and manipulating eukaryotic DNA without breaking it. Living

cells are lysed in 2 M salt to release structures which retain many of the morphological features of nuclei. These nucleoids contain naked nuclear DNA packaged within a flexible cage of RNA and protein. The linear DNA behaves as if it is unbroken and supercoiled and therefore it must be looped to make it <u>quasi</u>-circular[7-10]. Others who are probably working with similar structures, have confirmed this <u>quasi</u>-circularity[11-13].

Recently, enzymes (topoisomerases) which decrease and increase super-coiling [i.e. the untwisting enzyme from rat liver[14] and DNA gyrase from E.coli[15]] have been isolated. Using these enzymes we have twisted and untwisted nucleoid DNA to alter the degree of exposure of the bases and then transcribed the allomorphic templates using both plant and bacterial polymerases. We find that supertwisting induced by DNA gyrase stimulates dramatically transcription by polymerase II from wheat germ.

MATERIALS AND METHODS

Templates

Templates were obtained as follows:- PM2 DNA (containing $>$ 80% form I) was bought from Boehringer. Untwisted PM2 DNA was prepared[15] using the untwisting enzyme purified from rat liver[14] (fraction IV); single-stranded PM2 DNA was made by heat-denaturing DNA restricted with the endonuclease HpaII (Boehringer)[29]; HeLa nucleoids were prepared as described[8,9]. PM2 DNA and nucleoids were γ-irradiated[30] with 400 and 163.2 J Kg^{-1} respectively – doses which are sufficient to nick $>$ 95% of the circles or quasi-circles[8].

Transcription

Methods for determining maximal rates of RNA synthesis have been described[28]. Prior to transcription, templates (nucleoids at 0.3 x 10^6/ml, pure DNA at 7 µg/ml) were incubated without or with 90 units of gyrase (phosphocellulose fraction; units as defined by Gellert et al.[27]) at 23°C for 30 min in 209 µl containing 24 mM KCl, 40 mM potassium phos-phate, 25 mM Tris (pH 7.2) 6 mM MgCl$_2$, 360 µg/ml bovine serum albumin, 1.8 mM spermidine, 1.4 mM ATP, 5 mM dithiothreitol, 0.14 mM EDTA and 350 µg/ml tRNA. Nucleoid assays also contained 50 mM NaCl. The mixture was then diluted to 244 µl for transcription by addition of nucleotides and RNA polymerase (E.C.2.7.7.6) to give final concentrations of 0.4 mM ATP, GTP and CTP, 0.05 mM UTP, 100 µCi/ml [^{3}H]UTP and either E. coli RNA polymerase (Boehringer; 3 units/ml) or RNA polymerase II from wheat germ (Miles; 2 units/ml). Incubation was continued at 23°C, 20 µl

samples taken and acid-insoluble radioactivity counted.

Twisting, untwisting and nicking assays using nucleoids

Procedures for isolating HeLa nucleoids from cells growing in suspension and for handling, counting, γ-irradiating and monitoring by fluorometry the superhelical status of their DNA have been described[8,9]. Nucleoids isolated in 1.95 M NaCl were diluted to a final concentration of 1×10^6 nucleoids/ml (i.e. 12 µg DNA/ml). Nicking and untwisting assays were conducted for 15 min at $37^{\circ}C$ in 0.2 M NaCl, 10 mM Tris (pH 8.0) supplemented with 5 mM $MgCl_2$ and 1 mM EDTA respectively. Gyrase was assayed in 0.1 M NaCl, 20 mM potassium phosphate, 25 mM Tris (pH 7.2), 6 mM $MgCl_2$, 360 µg/ml bovine serum albumin, 1.8 mM spermidine, 1.4 mM ATP, 5 mM dithiothreitol, 0.14 mM EDTA: incubations were for 15 min at $23^{\circ}C$. (In some assays, ATP was omitted or coumermycin (30 µg/ml) added). Reactions were started by the addition of enzyme and stopped by adding a concentrated salt solution to give a final concentration of 0.2×10^6 nucleoids/ml in 2.0 M NaCl. Some of the nucleoids were irradiated (9.6 J kg^{-1}), ethidium added to a final concentration of 8 µg/ml and the amount of dye bound measured by fluorometry. (In some untwisting assays ethidium (8 µg/ml) was also present during the incubation). We define one unit of nicking or gyrase activity (assayed in the absence of ethidium) as the amount of activity that halves the difference in ethidium binding by unirradiated and irradiated nucleoids. One unit of untwisting activity is defined similarly except that the enzyme is assayed in the presence of ethidium. The various activities can be differentiated as indicated in Table I.

Table 1. **Assay of nicking, twisting and untwisting activities using nucleoids**

Enzyme activity	Ethidium (8µg/ml) added before or after reaction	Amount of ethidium bound by unirra-diated nucleoids	Difference in binding to unirradiated and irradiated nucleoids
Nicking	Before	Increases	Decreases
"	After	Increases	Decreases
Untwisting	Before	Increases	Decreases
"	After	Slightly decreases	Increases
Gyrase + ATP	After	Increases	Decreases
" − ATP	After	Slightly decreases	Increases
" + ATP + coumermycin	After	Slightly decreases	Increases

RESULTS

Twisting and untwisting assays

    The untwisting enzyme and gyrase were purified and assayed using
published procedures[14,15]. We have also developed assays using HeLa
nucleoids based on a procedure for detecting single-strand breaks in DNA[8].
At high concentrations the binding of the intercalating dye, ethidium, to
circular DNA induces supercoiling of sense opposite to that initially
present. Less ethidium binds to such a positively supertwisted molecule
than to its broken and so relaxed counterpart[4]. [This is the basis of the
widely used method for separating supercoiled from nicked DNA in caesium
chloride density gradients]. Like circles of pure DNA in 8 μg/ml ethidium,
unirradiated nucleoids bind less dye than γ-irradiated nucleoids with
relaxed quasi-circles[8]. Any increase or decrease in supercoiling alters
ethidium binding, which we measure fluorometrically. Nicking, twisting
and untwisting activities are all detected by modifying the basic procedure.
The method is rapid, sensitive and the template - nucleoid DNA - can be
prepared easily in milligram quantities from living cells in one step in
less than an hour[9].

    We incubate unirradiated and irradiated nucleoids with enzymes, add dye
before or after incubation and measure the amount bound by fluorometry.
This comparative approach using irradiated and unirradiated nucleoids allows
us to distinguish specific effects on supercoiling from any non-specific
effects. [For example, if proteins bind to DNA without altering supercoiling
and block dye-binding sites, they do so equally to both kinds of template.
On the other hand, uncoiling induced by the histones or other unwinding
agents can be detected (Levin, J.M. and Cook, P.R. manuscript submitted
for publication). The very low radiation dose introduces so few nicks -
< 5% of PM2 DNA circles are nicked with this dose[8] - that the extra nicks
in irradiated nucleoids cannot contribute much to any effects on protein
binding]. In the absence of enzymes, more ethidium binds to the irradiated
nucleoids. In the presence of dye, any nicking or untwisting of the unirra-
diated nucleoids increases their ethidium binding to the level of irradiated
nucleoids (Table 1). If ethidium is added after the reaction, nicking
again increases the amount bound but untwisting reduces it. This follows
because addition of the dye to the intact but untwisted product positively
supertwists it and such positive supercoiling is unfavourable to dye-
binding. Gyrase has the opposite effect to the untwisting enzyme; in the
presence of ATP - the introduction of further (negative) supercoiling

requires energy - the difference in dye-binding by the irradiated and
unirradiated nucleoids is decreased. The gyrase becomes an untwisting
(relaxing) enzyme in the absence of ATP or when the ATP-requiring subunit
is inactivated by the antibiotic, coumermycin. These general effects are
summarised in Table 1 and specific examples (i.e. nicking by irradiation,
twisting with gyrase and untwisting with gyrase without ATP) are given in
Table 2. We have confirmed our interpretation of these dye-binding studies
by electron microscopy. For example, nucleoid DNA spread for electron
microscopy using the Kleinschmidt procedure is extensively supercoiled
and pretreatment with the untwisting enzyme relaxes it[10].

Transcription of supercoiled PM2 DNA

    We first transcribed a viral DNA (i.e. from PM2). Superhelical circles
are transcribed faster by the RNA polymerase from E. coli than circles
nicked with γ -rays (Table 3, 1a and d) or cut once with a restriction
endonuclease (results not shown). However pretreatment of superhelical PM2
with gyrase slightly depresses transcription (Table 3, 1b). This is not
surprising since PM2 DNA is naturally highly-supercoiled[16] and overtwisted
templates are known to be inefficiently transcribed by the bacterial
enzyme[17]. In sharp contrast, gyration stimulates sixfold transcription by
RNA polymerase II from wheat germ (Table 3, 1b): the plant enzyme prefers
the overtwisted template. Several controls confirm that it is the over-
twisting that stimulates transcription: coumermycin, an antibiotic that
specifically inhibits the overtwisting activity of gyrase so converting
it into a relaxing enzyme[18], or nicking with γ-rays abolish the

Table 2. The effects of gyrase on supercoiling in nucleoids.

Treatment	Relative binding (%)	% difference in relative binding
Unirradiated	100)	20
Irradiated	120)	
Unirradiated + gyrase + ATP	120)	2
Irradiated + gyrase + ATP	122)	
Unirradiated + gyrase - ATP	108)	12
Irradiated + gyrase - ATP	120)	

The amounts of ethidium bound to nucleoids treated as described for the
gyrase assay in Table 1 are expressed as a percentage of the amounts
bound by untreated unirradiated nucleoids. Ethidium was added after
treatment with 120 units/ml of the phosphocellulose fraction of E. coli
gyrase[15]. (One unit is defined by Gellert et al[27]). There is some
nicking activity present in the gyrase which is detectable in the absence
of ATP. (No tRNA - a nuclease inhibitor - was used in this assay).

Table 3. The rates of RNA synthesis directed by allomorphic templates.

Template	Pre-treatment	Rate of synthesis			
		Bacterial polymerase		Plant polymerase	
		Absolute	Relative	Absolute	Relative
1a Supercoiled PM2		3.5	1.00	0.1	1.0
b Supercoiled PM2	+ gyrase	3.0	0.86	0.6	6.0
c Supercoiled PM2	+ gyrase + coumermycin	0.5	0.14	0.2	2.0
d Supercoiled PM2, irradiated		0.7	0.20	0.07	0.7
e Supercoiled PM2, irradiated + gyrase		0.4	0.11	0.06	0.6
f Single-stranded PM2		-	-	2.0	20.0
2a Untwisted PM2		0.6	1.0	0.02	1.0
b Untwisted PM2	+ gyrase	2.0	3.33	0.6	30.0
c Untwisted PM2	+ gyrase + coumermycin	0.2	0.33	0.03	1.5
d Untwisted PM2	, irradiated	0.7	1.17	0.03	1.5
e Untwisted PM2	, irradiated + gyrase	0.4	0.67	0.1	5.0
3a Nucleoids		1.3	1.0	0.03	1.0
b Nucleoids	+ gyrase	1.5	1.15	0.3	10.0
c Nucleoids	+ gyrase + coumermycin	1.3	1.0	0.08	2.7
d Nucleoids	, irradiated	1.3	1.0	0.03	1.0
e Nucleoids	, irradiated + gyrase	1.5	1.15	0.08	2.7

Methods for determining maximal rates of RNA synthesis are described in Materials and Methods. Absolute rates are expressed as pmoles UMP incorporated/20 µl sample/10 min. The gyrase concentrations used were sufficient to maximally supertwist the PM2 or nucleoid DNA (assayed using gels and fluorometrically, respectively).

stimulation (Table 3, 1c and e). As expected, single-stranded PM2 DNA is an excellent template for the plant enzyme (Table 3, 1f).

Transcription of untwisted PM2 DNA

Intact circles which have been relaxed by pretreatment with the untwisting (nicking-closing) enzyme from rat liver are as poor templates for both polymerases as are their counterparts which have been nicked by irradiation (Table 3, cf 1d and 2a) or cut once with a restriction endo-nuclease (results not shown). With this untwisted template, supertwisting with gyrase stimulates transcription by both polymerases (Fig. 1a; Table 3, 2b). The effect with the plant enzyme is dramatic, the rate of transcription increasing thirtyfold. Again the control experiments confirm that the stimulation is induced by the gyrase (Table 3, 2c-e).
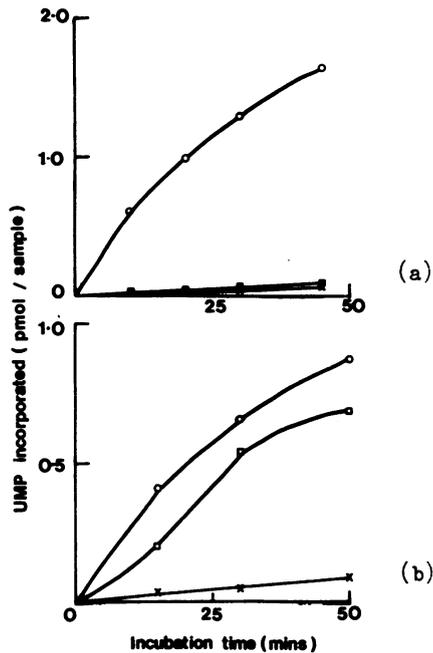
Figure 1. Gyrase stimulates transcription.
     Transcription assays were conducted as described in Materials and Methods. (a) Transcription of untwisted PM2 DNA by plant polymerase alone (X), pretreated with gyrase (0) or pretreated with gyrase and coumermycin (30 μg/ml) (☐). (b) Transcription of nucleoids by the plant polymerase alone (X), pretreated with gyrase (0) or treated with gyrase during transcription (i.e. polymerase and gyrase added simultaneously) (☐).

Transcription of nucleoids

     We next studied the effects of gyrase on transcription directed by nucleoids from HeLa cells. Since the quasi-circles in nucleoids are about 22 times the length of the viral circles, i.e. 2×10^5 base-pairs[8] and since only 1 nick releases all supercoiling from a quasi-circle, great care must be taken to suppress the effects of any contaminating nucleases. Both our gyrase preparation and the commercially-supplied wheat germ polymerase contained low levels of nucleases but their activity could be suppressed by the inhibitor tRNA and by conducting assays at $23^\circ C$. [Nuclease activity (i.e. nicking) was monitored both by gel electrophoresis[8] and by the more sensitive fluorometric assay].

     Pretreating nucleoids with gyrase hardly affects transcription by the

bacterial polymerase but increases tenfold transcription by the plant enzyme (Fig. 1b; Table 3, 3b). Again, control experiments using coumermycin and irradiated nucleoids confirm that the dramatic stimulation depends upon an active gyrase and an intact template (Table 3, 3b–e). The gyrase stimulates transcription after a slight lag when it is added to the nucleoids simultaneously with the polymerase (Fig. 1b).

DISCUSSION

Using DNA gyrase and the untwisting enzyme, we have prepared an allomorphic series of templates derived from 2 different superhelical DNAs – PM2 and the nuclear DNA of HeLa cells. We have then transcribed these templates using 2 different RNA polymerases – from wheat germ and E. coli. As others have found, both polymerases transcribe the naturally-occurring allomorphs at an equal or greater rate than their relaxed or nicked counterparts[19-21]. The degree of supercoiling in untreated PM2 or nucleoid DNA is about optimal for the bacterial polymerase. In contrast, it is sub-optimal for the plant enzyme since supertwisting with gyrase dramatically stimulates transcription to levels approaching those obtained with single-stranded DNA. RNA polymerase II must be even more dependent for efficient transcription on the free energy associated with negative supercoiling than is the E. coli polymerase, and it is for this reason that intact double-stranded but relaxed DNA is such a poor template for the wheat germ enzyme.

We have also compared the rates of transcription directed by nucleoids and equal weights of pure HeLa DNA (results not shown). As others have found[1], the plant enzyme transcribes pure DNA at rates which depend on the integrity of the DNA – the more ends and single-strand breaks, the higher the rate. As we can handle neither pure HeLa DNA nor that of untwisted nucleoids without breaking it – untwisted nucleoid DNA spills out of the cage – we are unable to isolate the stimulation induced by supercoiling from these other effects, but gyrated nucleoid DNA is probably transcribed thirty times faster than its relaxed but intact counterpart just like the superhelical allomorph of untwisted PM2 DNA. This highlights one great advantage of using nucleoids as a template for transcriptional studies. The ability to reconstruct rapidly complexes of histones and nucleoids without breaking their DNA is another (Levin, J.M. and Cook, P.R., manuscript submitted for publication). We hope now to go on to see whether the stimulation we see is specific in the sense that transcripts similar to

those found in vivo are synthesised.

In vivo, DNA is folded around histone cores to form nucleosomes[22] and we know that the isolated complex contains no free energy of supercoiling that can be released by the untwisting enzyme[23]. As a result, one current view is that there is unlikely to be a eukaryotic counterpart to the bacterial gyrase[24]. But it is attractive to suppose that a eukaryotic gyrase, by exposing the template surface and so stimulating transcription, determines the functional state of genes[25,26]. Furthermore, we have some suggestive evidence for a gyrase in HeLa cells; the DNA of nucleoids derived from cells grown in the presence of novobiocin - another specific inhibitor of the bacterial gyrase[18] - is less negatively supercoiled than that of untreated controls (Cook, P.R. and Brazell, I.A., unpublished observations).

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