

# Effect of *in vitro* transcription on cruciform stability

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## ABSTRACT

**We have investigated the effect of *in vitro* transcription on cruciform stability. Replicative form DNA of  $\phi$ X174 strain *ins6240*, containing a 48 bp synthetic palindrome in the J-F intercistronic region, was supercoiled *in vitro* to mean negative superhelical densities ( $\sigma$ ) ranging from 0 to 0.15. The presence of cruciforms was probed by limited digestion with the single-strand specific nuclease *Bal31*. The 48 bp palindrome was extruded at a mean  $\sigma = -0.05$ , but only after heating the DNA. An *in vitro* transcription reaction with *E. coli* RNA polymerase and [ $\alpha$ -<sup>32</sup>P]UTP gave identical transcripts with heated or unheated template DNA. The synthetic cruciform was stable upon binding of the RNA polymerase to the template, but it was destabilized upon movement of the transcription complex along the template. Transcription of unheated templates did not result in cruciform formation. We propose that cruciform structures in supercoiled template DNAs present no hindrance to RNA polymerase, and thus have no detectable effect on transcription elongation *in vitro*.**

## INTRODUCTION

The existence of supercoil induced non-B DNA secondary structures, and their potential role in the regulation of gene expression, has been discussed in several reviews (1,2,3,4). DNA supercoiling is an endergonic process and the stored free energy is thought to stabilize unusual DNA secondary structures (5) such as slippage sites (6,7), cruciforms (8,9) and Z DNA (10). The free energy of supercoiling can also be coupled to processes that induce unwinding of the DNA helix, such as transcription (11,12).

While the importance of secondary structure formation in RNA for the regulation of transcription termination, RNA processing, and turnover is well known (13,14), the significance of DNA secondary structure for the regulation of transcription is just emerging. Sequences capable of transition from B to Z DNA have been shown to induce early termination (15) or to act as a transcriptional block *in vitro* (16), at superhelicities that favor the B to Z transition. The introduction of a palindromic sequence within the -35 to +1 region of an *E. coli* promoter has been shown to prevent transcription in a supercoil dependent manner

(17,18). However, a direct effect of cruciforms on transcription elongation, such as that shown for B to Z transitions, has not been demonstrated. Recent findings that support the twin-supercoiled-domain model of RNA transcription point to a role of transcription in induction of DNA supercoiling and therefore in DNA secondary structure (19–24). To study the effect of transcription on cruciform stability, we have used a mutant of the genetically well defined genome of  $\phi$ X174. The mutant strain *ins6240* contains a 48 bp palindromic insert in the region between genes J and F (25). We have previously studied the structural alterations of wt and mutant replicative form (RF) DNAs in response to changes in supercoil, using single-strand specific nucleases as probes for cruciform formation (26). Herein we report the effects of *in vitro* transcription on cruciform stability, and the effect of cruciforms on the overall pattern of *in vitro* transcription of  $\phi$ X174 RF DNA.

## MATERIALS AND METHODS

### Enzymes and chemicals

*E. coli* RNA polymerase (RNAPol) was generously provided by R. Johnson (ECU School of Medicine). All enzymes were purchased from Bethesda Research Laboratories, U.S. Biochemical Corporation, or Boehringer Mannheim. Specialty reagents were from Sigma Chemical Company, and radionuclides from New England Nuclear.

### Bacterial and phage strains

Bacteriophage  $\phi$ X174 *am3* (amber mutation in the lysis gene) is referred to as wt. Mutant strain *ins6240* contains a 157 bp insert in the J-F region, which includes a 48 bp palindromic sequence. The construction of this strain was described in detail before (25). *E. coli* strain HF4714 contains an amber suppressor and was used as the permissive host (27). *E. coli* C was the non-permissive host.

### Preparation and *in vitro* supercoiling of $\phi$ X174 replicative form (RF) DNA

Procedures were carried out as described previously (26).

### Transcription assay

All reagents and glassware were sterile. Water for reagents was treated with 0.1% diethyl pyrocarbonate for 8 h at 4°. The

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transcription buffer contained 20 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 150 mM KCl, 0.1 mM dithiothreitol, 5 mM MgCl<sub>2</sub>, 0.5 mg/ml bovine serum albumin and 15% (v/v) glycerol. Reactions were carried out in a 30  $\mu$ l volume. Template DNA (0.3  $\mu$ g) was first incubated with *E. coli* RNAPol (0.5  $\mu$ g) for 1 min at 37° (RNAPol:DNA molar ratio of 5:1). Transcription was initiated by addition of 200  $\mu$ M each of ATP, CTP and GTP and 2.6  $\mu$ M of [ $\alpha$ -<sup>32</sup>P]UTP. Reinitiation was inhibited by addition of 10  $\mu$ M rifampin 30 s after initiation; 200  $\mu$ M UTP was added 5 min later. The reaction was stopped after 20 min by chilling on ice and ethanol precipitation (3 volumes of ethanol, 0.3 M Na acetate, 10  $\mu$ g *E. coli* tRNA as carrier). Precipitates were recovered by centrifugation, washed twice with ethanol, air dried, and processed for electrophoresis as described by Maniatis *et al.* (28).

### Electrophoresis of RNA

Agarose gels (1.25%) containing 2.2 M formaldehyde were prepared as described by Maniatis *et al.* (28), and run at 70 mAmps, until the tracking dye (bromophenol blue) migrated 14 to 15 cm. <sup>32</sup>P end-labeled restriction enzyme fragments of  $\phi$ X174 were denatured (100°, 2 min) and processed exactly as the transcripts before use as size markers. Gels were dried and exposed to Kodak  $\times$ Omat AR film.

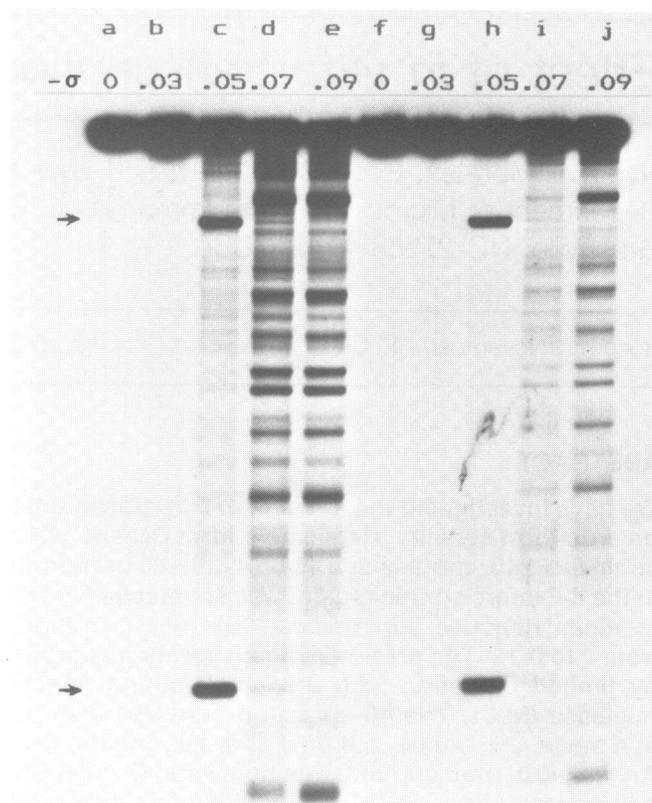
### Detection and mapping of cruciforms

The presence of cruciforms was probed with *Bal31* nuclease (29) as described previously (26). DNAs (3 to 5  $\mu$ g) in transcription buffer with or without *E. coli* RNAPol were incubated for 1 min at 37° with  $7 \times 10^{-3}$  units of *Bal31* nuclease. The concentration of the enzyme was such that only approximately 50% of the supercoiled templates were linearized in 1 min. After extraction with phenol and precipitation with ethanol, the DNA was digested to completion at the single *AvaII* site. The resulting fragments were labeled at the 5' end with T4 DNA kinase and [ $\gamma$ -<sup>32</sup>P]ATP and separated by electrophoresis on 1.4% agarose gels (26).

## RESULTS

### The synthetic palindrome forms a stable secondary structure in the presence of RNA polymerase

The 48 bp synthetic palindromic sequence of strain *ins6240*, once extruded into a cruciform, is very stable. The single-stranded loop region of the cruciform can be cleaved by the endonuclease action of *Bal31* (Müller, U.R. and Wilson, C.L., unpublished). The effect of the transcription buffer and RNAPol binding on cruciform stability was tested as follows. DNA from strain *ins6240* was supercoiled *in vitro* to different mean negative superhelical densities, heated at 65° for 30 min and cooled to room temperature, to allow for cruciform formation (26). The DNA samples were then incubated for 1 min at 37° in transcription buffer with or without RNAPol. Secondary structures were then probed by limited digestion with *Bal31*, followed by complete digestion at the unique *AvaII* site, as described in Methods. The reaction products were then end-labeled and separated on agarose gels. Each *Bal31* cleavage site should be represented by two fragments of characteristic size. The majority of the *Bal31* sensitive sites (either supercoil dependent or independent) of the  $\phi$ X174 wt genome have been mapped by this method (26). Cleavage at the J-F synthetic cruciform is represented by fragments 4,100 and 1,400 bp long.

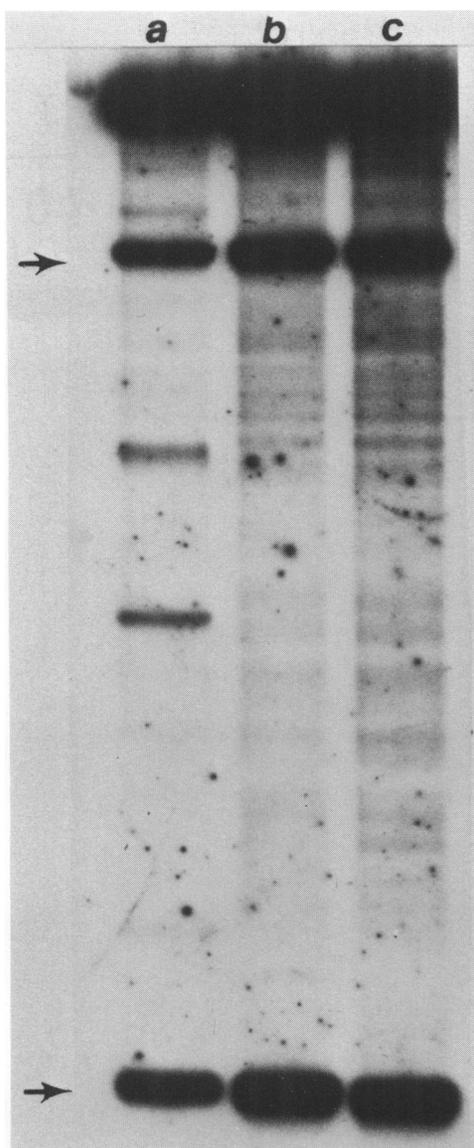


**Figure 1.** Effect of transcription buffer and RNA polymerase binding on DNA secondary structure. DNA from  $\phi$ X174 strain *ins6240* was supercoiled *in vitro*, heated at 65° for 30 min and allowed to cool slowly to room temperature. The DNA was then incubated for 1 min at 37° in transcription buffer without (lanes a to e) or with *E. coli* RNAPol (lanes f to j). The presence of cruciforms in the DNA was then probed with *Bal31* nuclease. After removal of the nuclease with phenol, the DNA was digested to completion with *AvaII*, end-labeled with <sup>32</sup>P and electrophoresed on a 1.4% agarose gel (26). The mean superhelical density ( $\sigma$ ) of the DNA is indicated for each lane. The arrows point to the 4,100 and 1,400 bp bands that correspond to the synthetic cruciform. Restriction fragments of  $\phi$ X174 RF DNA, end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP, were used as size markers (not shown).

Fig. 1, lane c shows two predominant fragments of that size appearing at a mean  $\sigma = -0.05$ , suggesting that the palindromic insert is extruded into the cruciform structure in transcription buffer. Additional competing structures appear at superhelicalities exceeding  $-0.05$ . Presence of the RNAPol (Fig. 1, lanes f to j) reduces the intensity of the bands somewhat, suggesting that the total number of structures (ie supercoil dependent *Bal31* cleavage sites) has been reduced. This would be expected according to the model of 'open complex' formation preceding transcription initiation, which predicts a decrease in superhelical density of the template (12). Nevertheless, the synthetic cruciform is the predominant structure at mean  $\sigma = -0.05$  (lane h). Furthermore, the cruciform is stable for at least 60 min after binding of the RNAPol (Fig. 2). A decrease in the migration during electrophoresis of the DNA template in the presence of the RNAPol (Fig. 3) indicates that the polymerase is actually bound to the template.

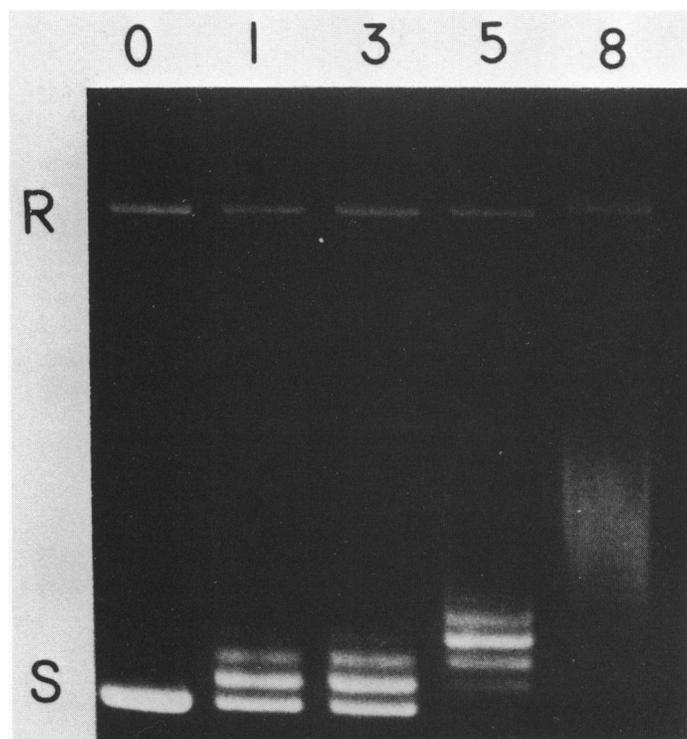
### The synthetic cruciform does not have a qualitative effect on transcription

Fig. 4A shows that the cruciform is present in a large percentage of heated (65°) DNA molecules with a mean  $\sigma = -0.05$ , but only in a small percentage of unheated (4°) DNA molecules.



**Figure 2.** Stability of the synthetic cruciform in the presence of RNA polymerase. Heated DNA ( $65^{\circ}$ ) from  $\phi$ X174 strain *ins6240* with a mean  $\sigma = -0.05$  was incubated in transcription buffer with RNAPol at  $37^{\circ}$ , for 1 (lane a), 30 (lane b) or 60 min (lane c). The presence of the cruciform was probed with *Bal31* as described in Methods and the resulting DNA fragments were electrophoresed as in Fig. 1. The arrows point to the 4,100 and 1,400 bp bands that correspond to the synthetic cruciform.

Nevertheless, the number and size of the transcripts obtained from heated ( $65^{\circ}$ ) or unheated ( $4^{\circ}$ ) templates (ie with or without the cruciform, respectively) are identical (Fig. 4B). Fig. 4C shows the location of the 3 known promoters in the  $\phi$ X174 genome, which are located upstream from the J-F intergenic region (ie the synthetic palindrome in *ins6240*). The *in vivo* active terminators are downstream from the palindrome (30,31). Mapping of the *in vitro* active termination sites has been attempted by several labs with different results (for summary see Figs. 1 in refs. 30 and 32). With the exception of possibly one terminator between  $P_A$  and  $P_B$ , they either overlap with *in vivo* active termination sites, or are located between  $T_J$  and  $T_F$  (not shown). Thus, most *in vivo* and *in vitro* transcripts are polycistronic and include the J-F region. Based on the distance between the promoters and the J-F region (Fig. 4C), and the size distribution

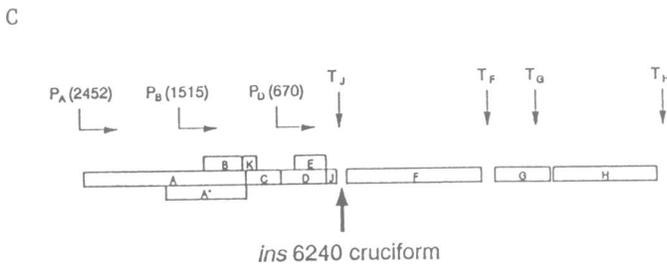
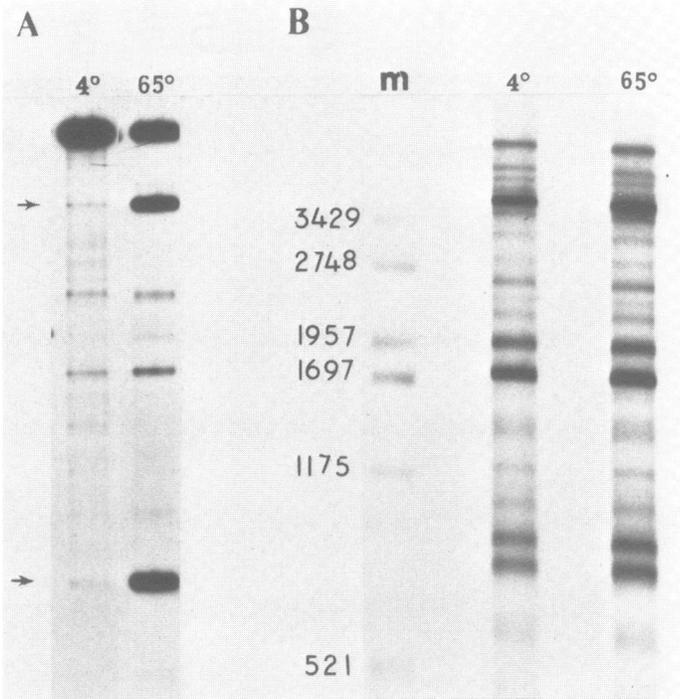


**Figure 3.** Effect of RNA polymerase binding on the migration of template DNA during electrophoresis. DNA from  $\phi$ X174 *ins6240* was incubated in transcription buffer with increasing concentrations of RNAPol at  $37^{\circ}$  for 1 min. The DNA was then electrophoresed on 1% agarose gels and stained with ethidium bromide (26). The RNAPol:DNA molar ratio is indicated for each lane. R: relaxed DNA. S: supercoiled DNA.

of our *in vitro* transcripts (Fig. 4B), we conclude that at least half of the transcripts include the synthetic palindrome. Mapping data support this conclusion (Morales, N.M. and Müller, U.R. manuscript in preparation). Similar results have been obtained with strains containing synthetic palindromes of different length and base composition (not shown). The present data therefore indicate that the transcription assay is functional, and that the presence of the synthetic cruciform in the pre-initiation complex does not have a qualitative effect on transcription.

#### Movement of the transcriptional complex along the template melts the extruded palindrome

The apparent inability of the synthetic palindrome to alter the pattern of transcription *in vitro* (Fig. 4B) could be due to melting out of the cruciforms by the transcription elongation complex. Evidence for this is shown in Fig. 5. Heated DNA from strain *ins6240* with a mean  $\sigma = -0.05$  was used as template in the transcription assay. Samples were probed with *Bal31* after binding of RNAPol in the absence of nucleotides and at various times after addition of nucleotides. Reinitiation was inhibited with rifampin. Under these conditions, transcription terminated in less than 20 min. The J-F cruciform was present in the pre-initiation complex (pi, lane 1'), but it was substantially diminished three minutes after the onset of transcription (lane 3'), as evidenced by the decrease in the intensity of the bands characteristic of the J-F cruciform. Not even a trace of *Bal31* cleavage at this structure or any of the other supercoil dependent recognition sites could be detected 10 (lane 10') or 40 min later (ie after transcription termination, lane 40'). Since we have shown above that the

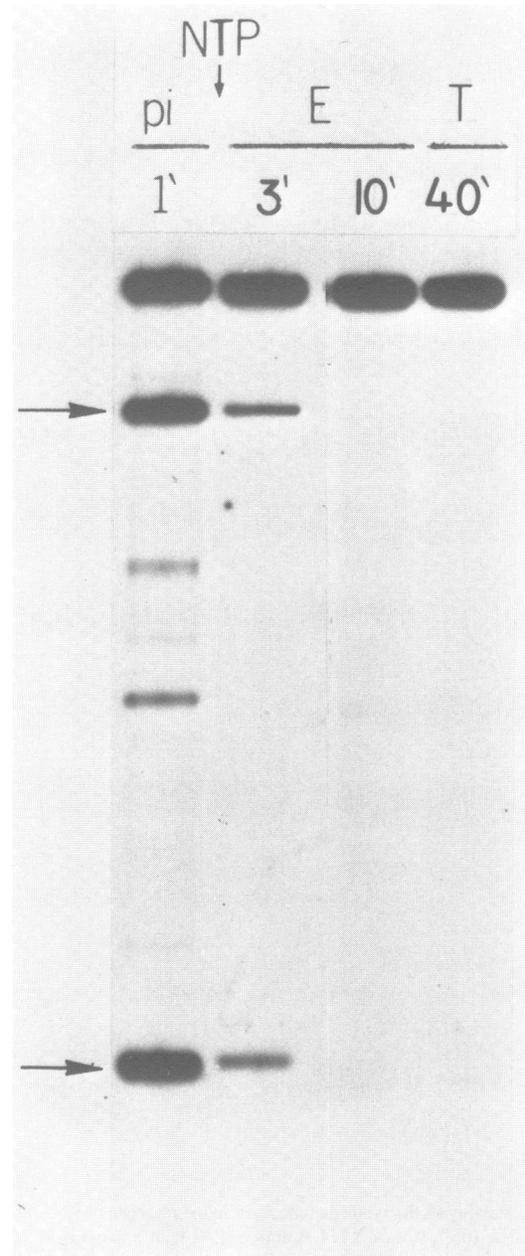


**Figure 4.** Effect of cruciform formation on *in vitro* transcription. **A.** Electrophoresis of DNA probed with *Bal31*. DNA from  $\phi$ X174 strain *ins6240* with a mean  $\sigma = -0.05$  was incubated at 4° or at 65° for 30 min and the formation of the synthetic cruciform was probed with *Bal31*, as described for Fig. 1. The arrows point to the 4,100 and 1,400 bp fragments characteristic of the synthetic cruciform. **B.** Electrophoresis of RNA transcribed *in vitro*. Unheated (4°) or heated (65°) DNA was used as template in an *in vitro* transcription assay with *E. coli* RNAPol and [ $\alpha$ -<sup>32</sup>P]UTP, as described in Methods. The transcripts were separated by electrophoresis on 1.25% agarose-2.2 M formaldehyde gels (28). The size of  $\phi$ X174 restriction enzyme fragments used as markers (m) is indicated. **C.** Genetic map of the circular  $\phi$ X174 genome, linearized at the intercistronic region between genes H and A. The location and the direction of transcription of the 3 known promoters (P), and the location of the *in vivo* active terminators (T) is indicated by arrows, and was redrawn from Fig.1 in ref. (31). The distance in bp between each promoter and the synthetic palindrome in strain *ins6240* (arrow) is indicated in brackets.

cruciform was stable for at least 60 min in the absence of nucleotide triphosphates, but under otherwise identical conditions (Fig. 2), we conclude that incorporation of nucleotides (ie movement of the transcription complex along the template) was responsible for loss of the cruciform.

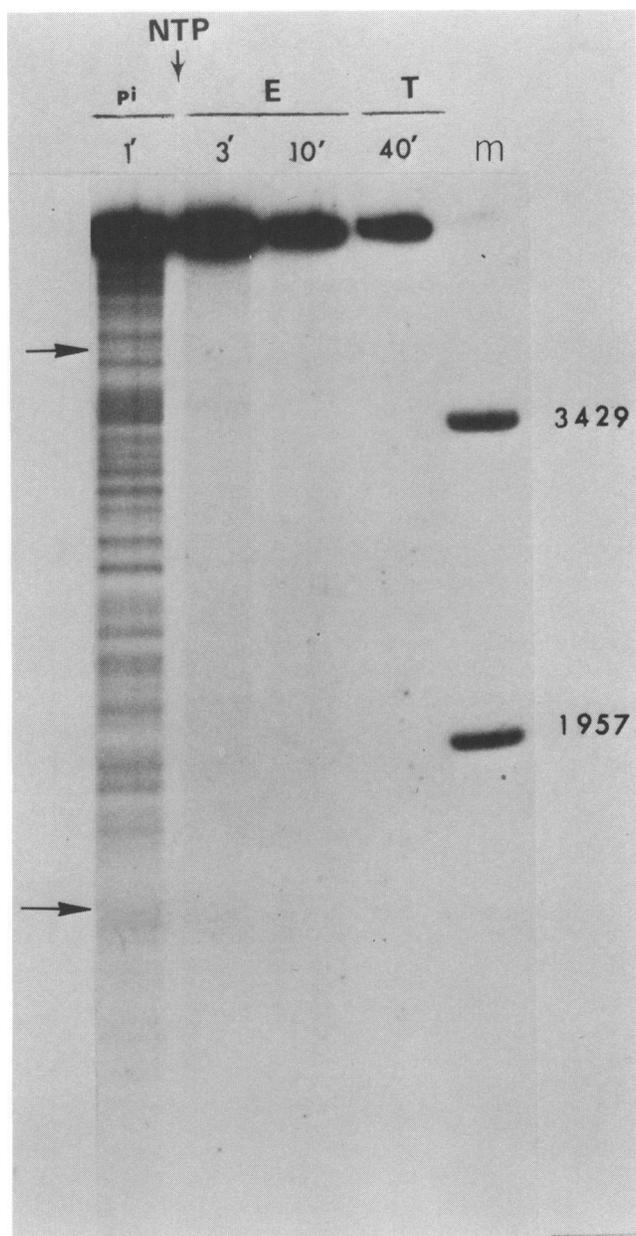
**Unwinding of the DNA by RNA polymerase does not result in cruciform formation**

It has been suggested that the unwinding of the DNA by the polymerase during transcription may overcome the energy barriers of cruciform formation and actually favor cruciform extrusion (16,33). This hypothesis was tested in our system using as template unheated DNA, where the inserted palindrome was



**Figure 5.** Effect of transcription elongation on cruciform stability. Heated DNA (65°) from  $\phi$ X174 strain *ins6240* with a mean  $\sigma = -0.05$  was incubated in transcription buffer with RNAPol at 37° for 1 min. Transcription was then initiated by addition of nucleotide triphosphates (NTP). The presence of cruciforms was assayed with *Bal31*, followed by electrophoresis (see Methods and legend to Fig. 1) in the pre-initiation complex (pi, 1') and 3, 10 or 40 min (lanes 3', 10', and 40' respectively) after addition of NTP. The arrows point to the 4,100 and 1,400 bp fragments characteristic of the synthetic cruciform. E: elongation. T: termination.

extruded in only a small fraction of the molecules in the DNA-RNAPol pre-initiation complex (Fig. 6, lane a). No additional extrusion could be observed either at 3, 10, or 40 min after the initiation of transcription (lanes 3', 10', and 40', respectively). On the contrary, overexposure of the autoradiograph revealed that transcription had resulted in disappearance not only of the bands characteristic of the inserted cruciform, but of all other supercoil dependent structures as well. These results suggest that movement of the transcription complex along the template in only one direction does not aid in cruciform formation, but appears to destabilize such structures.



**Figure 6.** Effect of transcription on cruciform formation. Unheated DNA from  $\phi$ X174 strain *ins6240* with a mean  $\sigma = -0.05$  was incubated in transcription buffer with RNAPol at  $37^\circ$  for 1 min. Transcription was initiated by addition of nucleotide triphosphates (NTP). The presence of cruciforms was assayed with *Bal31*, followed by electrophoresis (see Methods and legend to Fig. 1) in the pre-initiation complex (pi) and 3, 10 or 40 min (lanes 3', 10', and 40' respectively) after addition of NTP. E: elongation. T: termination. The size of restriction fragment markers from  $\phi$ X174 RF DNA (m) is indicated on the right. The arrows point to the position of the expected fragments characteristic of the synthetic cruciform.

## DISCUSSION

The purpose of this work was to study the reciprocal effects of transcription and DNA secondary structure *in vitro*. We have used  $\phi$ X174 DNA as template, since it is genetically and physically well defined, and the structural changes that occur in response to superhelical stress have been described (26). In addition, a variety of mutants with palindromic and non-palindromic insertions into the J-F intercistronic region are available. Furthermore, the system allows the evaluation of the

effects of the inserted sequence in interaction with other naturally occurring secondary structures.

Using a template DNA with a 48 bp synthetic palindrome and nuclease *Bal31* as probe for cruciform formation, we show that the inserted palindrome is extruded under the ionic conditions of the transcription assay, from heated templates with a mean  $\sigma = -0.05$  (Fig. 1). Furthermore, this cruciform is stable for at least 60 min after the formation of the 'open complex' between the template and the RNAPol (Fig. 2). Yet, the presence of the synthetic cruciform does not have a qualitative effect on *in vitro* transcription of  $\phi$ X174 DNA (Fig. 4B). This lack of a qualitative effect is most likely the result of the melting of the structure by movement of the transcription complex along the template (Fig. 5). Moreover, transcription does not result in extrusion of the 48 bp palindrome from unheated templates (Fig. 6), even though the overall superhelicity of the template (mean  $\sigma = -0.05$ ) should be sufficient for its stabilization (Fig. 1).

The twin-domain model of transcriptional supercoiling predicts the formation of positive supercoils in front of the transcription complex, and the generation of negative supercoils behind it (21-24). How this effects the local superhelical density and the overall structure of the genome depends on many different factors *in vivo*, but is much simpler in an *in vitro* system. Other than multiple transcription complexes that are moving toward or away from each other, there are no other large macromolecules or membrane attachment sites that may inhibit the circular diffusional pathway that should lead to cancellation of the positive and negative supercoils. The  $\phi$ X system is even simpler, since all promoters fire in the same direction (Fig. 4C). Thus, our data can be explained by a collapse of cruciform structures into the B-helix due to the positive supercoil (ie local reduction of negative superhelicity) of the advancing transcription complex. But these structures are not regenerated in the wake of the transcription ensemble, even though the local negative superhelicity has been increased. A likely explanation for this is a cancellation of the extra negative supercoils formed behind one transcription complex by the positive supercoils generated in front of an advancing second complex. The movement of RNAPol away from the promoter may also allow binding of additional RNAPol molecules, which would reduce the net superhelical density of the template during transcription elongation, and may therefore destabilize all cruciforms.

While multiple closely following transcription complexes may account for the absence of cruciforms during transcription elongation, the absence of any discernable structures after all transcripts are terminated (Fig. 5, lane 40') remains to be explained. Fig. 6 (lane 1') shows that there are many small naturally occurring cruciforms and other non-B DNA secondary structures possible in the  $\phi$ X RF DNA (also see ref. 26). At physiological temperatures the kinetic barriers for extrusion of these structures is sufficiently low, such that they can act as temporary sinks of the available supercoil energy. Heating of this DNA diminishes most of these structures (Fig. 1, lane c), and we have speculated previously that a supra-structure (possibly a toroidal form) exists, which is yet a better sink for the available supercoil energy. Only large synthetic palindromes (such as the one in *ins6240*) can store sufficient energy (ie are stable enough) to compete with this supra-structure (Fig. 1, lane c). However, both the large synthetic structures, as well as the hypothetical supra-structure, have kinetic barriers that prevent their formation at physiological temperatures, which allows the extrusion of naturally occurring palindromes. Our data then suggest, that melting the DNA during transcription overcomes the kinetic

barriers for formation of the supra-structure in its wake, which outcompetes the natural cruciforms (Fig. 6, lanes 3' to 40'). However, this process does not lower the kinetic barrier sufficiently to allow extrusion of large synthetic cruciforms.

## ACKNOWLEDGEMENTS

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