Template Supercoiling by a Chimera of Yeast *GAL4* Protein and Phage T7 RNA Polymerase

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Fusion of the DNA-binding domain of yeast *GAL4* protein to the amino terminus of bacteriophage T7 RNA polymerase yields a chimera that retains the characteristics of its components. The presence of the *GAL4* peptide allows the chimeric enzyme to anchor itself on the DNA template, and this anchoring in turn drives the formation of a supercoiled DNA loop, in linear or circular templates, when RNA synthesis at the polymerase site forces a translocation of the DNA relative to the site. Nonspecific interaction between the chimeric enzyme and DNA appears to be sufficient to effect supercoiling during transcription. Transcription by the chimeric polymerase is strictly dependent on the presence of a T7 promoter; thus it provides a tool in vitro and in vivo for specifically supercoiling DNA segments containing T7 promoter sequences.

THERE IS NOW SUBSTANTIAL EVIDENCE THAT THE TRANscription process may generate positive supercoils in the DNA template ahead of the advancing polymerase and negative supercoils in the DNA behind it (*1, 2*). This simultaneous formation of oppositely supercoiled domains is a consequence of the helical geometry of the DNA template; it occurs whenever the transcriptional machinery and macromolecules associated with it cannot encircle the DNA template as transcription proceeds, and thus the DNA must be rotated around its helical axis in concert with the translocation of the transcriptional ensemble (*1, 2*). In a living cell, the generation of supercoiled domains by transcription is accompanied by the relaxation of these domains by the DNA topoisomerases; oppositely supercoiled domains may also neutralize each other through diffusional pathways (*1*).

The detailed mechanics of this supercoiling process are yet to be elucidated. Two types of models have been postulated about conditions that might lead to template supercoiling during transcription (*1*). It is plausible that in the cellular milieu, rotating the DNA around its axis is subject to a resistance lower than that encountered by the circling transcriptional ensemble, especially for a long transcript. Alternatively, the transcriptional ensemble might be anchored to a large cellular structure or to the DNA itself; this anchoring would prevent the circling of the transcriptional ensemble around the DNA.

Among the various situations to which the anchoring model might apply, two appear to be of particular interest. One involves the transcription of membrane protein genes in prokaryotes. Because transcription and translation proceed simultaneously in prokaryotes, the NH_2-terminal portion of a membrane protein being synthesized from its message might, at the same time, become anchored on the membrane (*1*). This postulate is supported by data on the transcription of the *tetA* gene in pBR322 (*3*).

In the other model, the RNA polymerase R is assumed to be anchored to its template through interactions with a DNA binding factor X. In such a case, the DNA can be viewed as being threaded through the polymerase as transcription proceeds, with its helical geometry forcing it to rotate at the same time. The formation of a supercoiled loop between R and X is thus expected; in the case of a covalently closed circular template, a negatively supercoiled loop behind the polymerase and a positively supercoiled loop ahead of it would form simultaneously (*1, 4*).

It is well recognized that before the initiation of RNA synthesis, the transcriptional machinery is often in contact with DNA-bound regulatory factors (*5*). Little is known, however, about the events following the initiation of transcription. It is not known, for example, whether template supercoiling occurs in such a case, as one would predict. Neither is it clear whether the various protein-protein or protein-DNA contacts are maintained after the initiation of transcription, and if so for how long.

In order to address such questions, we have constructed a chimeric enzyme, in which the NH_2-terminal 148 amino acids of the yeast *GAL4* gene product, which contains both the DNA-binding and protein-protein dimerization motifs of the protein (*6*), is fused to the NH_2-terminus of phage T7 RNA polymerase. We show here that the fusion protein remains active as a T7 promoter-specific polymerase and retains the DNA sequence specificity of the *GAL4* protein (which we refer to as Gal4). Our studies show that anchoring of the chimeric enzyme, through specific or nonspecific interactions between its DNA-binding motifs and the DNA template, can lead to supercoiling of a circular or linear template. The construction of the chimeric RNA polymerase thus provides a simple system for dissecting the kinematics of transcription, and its use as a sequence-specific DNA supercoiling enzyme could range from the preparation of positively supercoiled DNA's in vitro, to testing the physiological consequences of localized DNA supercoiling in vivo.

Enzymatic and DNA-binding properties of the chimeric protein. The plasmid pEAO1 (Fig. 1) was constructed for the overexpression of the yeast Gal4 and phage T7 RNA polymerase fusion protein, which we term Gal4-T7 RNAP (*7*). In the chimera, all but the first two amino acids of phage T7 RNA polymerase are linked to the NH_2-terminal 148 amino acids of yeast Gal4 through a stretch of ten amino acids Pro-Ser-Thr-Ser-Arg-Gly-Gly-Pro-Val-Pro, which are encoded by a "polylinker" sequence introduced during the cloning of the fusion protein.

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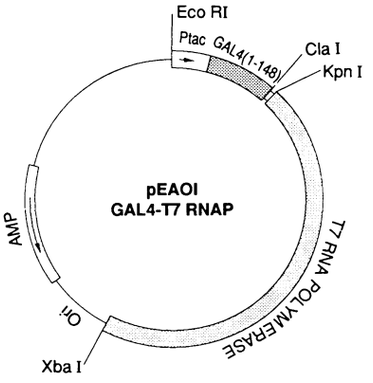

Fig. 1. Map of pEAO1, a plasmid constructed for the overexpression of the Gal4-T7 RNA polymerase fusion protein. The segment from the inducible *E. coli* promoter Ptac to the Cla I site (in the clockwise direction) was derived from a Gal4 expression plasmid pLPK76-7 (*6*). The segment from the Kpn I site going clockwise to the Xba I site was derived from p3695 (*7*). The Kpn I to Hind III segment of p3695 contains sequences encoding the phage polymerase; the Hind III site was repaired following digestion with the restriction enzyme, and the repaired end was abutted to a repaired Xba I end to recreate the Xba I site shown. The short segment from Cla I to Kpn I was derived from the polylinker region of pBluescript (Stratagene Cloning Systems, La Jolla, California). The fusion protein is thus encoded by, in sequence, the first 148 codons of the yeast GAL4 protein, a stretch encoding Pro-Ser-Thr-Ser-Arg-Gly-Gly-Pro-Val-Pro, which was introduced during the various cloning steps, followed by the third codon to the last codon of T7 RNA polymerase. The rest of pEAO1 from Xba I to Ptac was also derived from pLPK 76-7; this region contains a transcription terminator, and the replication origin (*Ori*) and the β-lactamase coding region (*Amp*) of pBR322.

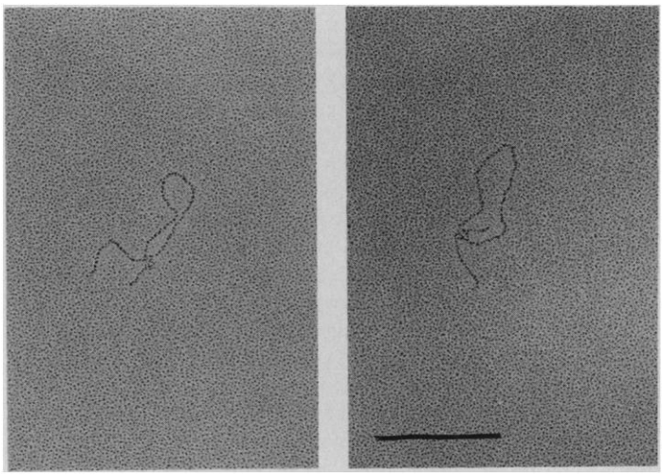

Fig. 2. Electron micrographs demonstrating the simultaneous binding of Gal4-T7 RNAP to a Gal4 cognate site and a T7 promoter. A 25-μl incubation mixture containing 95 ng of the fusion protein and 0.6 μg of DNA, in 20 mM Hepes buffer, pH 7.5, 7 mM $MgCl_2$, 70 mM NaCl, and 0.1 mM EDTA, was incubated at 32°C for 30 minutes. Glutaraldehyde was added to 0.6 percent final concentration, and, after 15 minutes at 37°C, ammonium acetate and glycerol were added sequentially to 100 mM and 50 percent (v/v), respectively. The mixture was immediately sprayed with an atomizer onto freshly cleaved mica, dried at reduced pressure, and rotary-shadowed with platinum at an angle of 7°. A carbon film was then deposited vertically on the metal-shadowed surface, and this carbon-coated replica was subsequently floated off the mica onto distilled water. Each replica was picked up on a 400-mesh copper grid and examined at 80 kV in a JEOL CXII electron microscope (*18*). Contour lengths of individual molecules were measured with a Kurta digitizing tablet attached to an IBM PC computer (*19*). Microscopy was also carried out with samples containing purified T7 RNA polymerase (*7*) instead of the fusion protein. Two plasmid DNA's were used in these experiments. pEAO14 was constructed from a pBlueScript backbone, in which the 122-bp Ssp I–Ssp I fragment was replaced with an 85-bp insert containing three tandemly positioned Gal4-binding sites (*8*). Plasmid pEAO16 is of the same basic construction, except that the Ssp I–Ssp I fragment was replaced by a 59-bp Hind III–Eco RI insert from pMH100 (*19*); this 59-bp fragment contained a single Gal4 cognate site. The bar represents 130 nm.

The purified fusion protein is active as an RNA polymerase with DNA templates containing phage T7 promoter sequences, such as pGEM3 (Promega), but not with templates without a phage T7 promoter, such as pBR322. Electron microscopic studies show that the fusion protein can simultaneously interact with a Gal4-binding site and a phage T7 promoter. In one series of experiments, a plasmid pEAO14 was cut with two restriction endonucleases Xmn I and Kpn I to give a 1.04-kb and a 1.89-kb fragment. The shorter fragment contained a cluster of three Gal4-binding 17-bp sequences (*8*), the center of the cluster being 250 bp from the Xmn I end. A T7 promoter is present on the same fragment about 120 bp from the Kpn I end. The longer fragment contained neither Gal4-binding sites nor T7 promoter sequences. On incubation with the fusion protein and fixation with glutaraldehyde, the macromolecules were adsorbed to freshly cleaved mica and were then processed for electron microscopy.

Approximately 20 percent of the shorter DNA molecules contained a loop (Fig. 2). Contour length measurements of 21 looped molecules gave a mean loop size ± standard deviation of 746 ± 66 bp. The lengths of the two ends of each molecule fell into two size classes, with mean values of 193 ± 28 bp and 91 ± 16 bp, respectively. These results are in good agreement with those expected for simultaneous binding of a fusion protein to a Gal4-binding site and a T7 promoter, located 250 and 120 bp from the ends, respectively, with a distance of about 660 bp in between. Looped DNA molecules were rarely seen for the 1.89-kb fragment in the same mixture; for the few that showed a crossover, the sizes of the loops varied greatly. Furthermore, the visible mass at each of the crossovers of the looped molecules shown in Fig. 2, which is most likely the fusion protein, was absent at the crossovers in the 1.89-kb fragment. In control experiments with purified T7 RNA polymerase instead of the fusion protein, no looped molecules similar to those shown in Fig. 2 was seen.

A second series of experiments was performed with a plasmid pEAO16, which is identical to pEAO14, except that a single Gal4-binding site has replaced the cluster of Gal4-binding sites in pEAO14. Looped molecules similar to those described above were again observed for the shorter Xmn I–Kpn I fragment containing Gal4-binding site and T7 promoter, but not for the longer fragment.

Induction of DNA supercoiling by transcription with the fusion protein. Two separate experiments show that transcription by Gal4-T7 RNAP induces DNA supercoiling. In the first experiment, relaxed pEAO17 DNA, which contains one T7 promoter and one Gal4-binding site 550 bp upstream, was incubated with the fusion protein in the presence of all four nucleoside triphosphates and *Escherichia coli* DNA topoisomerase I. Because the bacterial topoisomerase specifically relaxes negative supercoils (*9*), in its presence the simultaneous generation of two oppositely supercoiled domains by the fusion protein leads to a net accumulation of positive supercoils in the plasmid DNA (*1, 2*).

When a relaxed and covalently closed DNA was incubated with the fusion protein and *E. coli* DNA topoisomerase I, essentially all input DNA was converted to the supercoiled form (Fig. 3, lanes 1 and 2); the band in lane 2 with an electrophoretic mobility close to that of the untreated DNA contained nicked DNA rings, which were present in the input DNA.

If supercoiling of the DNA template by the chimera is driven by the translocation of the DNA-anchored protein, we would predict that template supercoiling should be dependent on RNA synthesis but not on the length of the nascent RNA. When ribonuclease (RNase) was added to the incubation mixture to degrade the RNA as it was being synthesized, supercoiling of the input DNA was largely unaffected (Fig. 3, lane 3). Assays for retention of labeled

RNA on DE-81 paper (*10*) confirmed that newly synthesized RNA was indeed degraded in the presence of RNase A. In contrast, when one or more of the four triphosphates was omitted in the transcription mixture, little supercoiling of the input DNA was detectable; reducing UTP (uridine triphosphate) concentration from 400 μM (Fig. 3, lanes 2 and 3) to 4 μM (Fig. 3, lane 4), whereas keeping the other three triphosphates at 400 μM each, also reduced the extent of template supercoiling. It is known that optimal transcription by T7 RNA polymerase requires a high UTP concentration for the suppression of abortive initiation (*11*).

When the fusion protein was substituted by purified T7 RNA polymerase, little supercoiling was seen (Fig. 3, lane 5). Because the specific activity of the phage polymerase preparation was about ten times higher than that of the fusion protein, the amount of the enzyme in the incubation mixture used in Fig. 3, lane 5, was ten times less than the amount of the fusion protein used in the incubation mixtures used in Fig. 3, lanes 2 and 3. There was little accumulation of positive supercoils in the template in the presence of *E. coli* DNA topoisomerase I and RNase, however, even when the amount of T7 RNA polymerase in the Fig. 3, lane 5, sample was ten times higher.

To demonstrate that template supercoiling by the fusion protein requires the presence of the DNA-binding domain of Gal4 and phage polymerase in a single protein, we added purified Gal4(1–147), the NH_2-terminal 147 amino acids of Gal4 (*12*), to the incubation mixture containing T7 RNA polymerase. Increasing amounts of Gal4(1–147) had little effect on template supercoiling (Fig. 3, lanes 6 to 9).

To confirm that positive rather than negative supercoils were accumulated in the DNA templates when transcription with Gal4-T7 RNAP was carried out in the presence of *E. coli* DNA topoisomerase I, we analyzed a fraction of the sample used in lane 2 of Fig. 3 by two-dimensional gel electrophoresis. Most of the DNA was indeed in the positively supercoiled form [compare the pattern shown in Fig. 4A with that for a mixture of negatively supercoiled topoisomers shown in Fig. 4C; see reference (*13*) for detailed interpretations of the two-dimensional patterns]. For example, a part of the sample run in lane 5 of Fig. 3, which was obtained by transcription of pEAO17 with T7 polymerase instead of the fusion protein in the presence of *E. coli* DNA topoisomerase I, was also analyzed on the same two-dimensional gel (Fig. 4B). Here most of the DNA migrated with mobilities identical to those of the topoisomers of the relaxed input DNA; only a faint streak extending downward from the bulk DNA was visible, demonstrating again that there was a much lower level of template supercoiling when the phage polymerase rather than the fusion protein was used (*2*).

Electron microscopy was also used to view the generation of a supercoiled loop by transcription of a linear DNA with the fusion protein. The plasmid pEAO18 was cut with Hind III restriction endonuclease to give a 3.2-kb linear molecule with a Gal4 cognate site very close to one end and two T7 promoters located about 600 bp from each end and pointing inward. After the DNA was incubated with the fusion protein in the presence or absence of all four ribonucleoside triphosphates, glutaraldehyde was added to fix the nucleoprotein complex, and the fixed sample was processed for electron microscopy. Molecules with tightly coiled loops were seen only after incubation of the linear DNA with the fusion protein in the presence of all four triphosphates (see Fig. 5 for examples).

Anchoring of the chimera through nonspecific binding to DNA. In designing the fusion polymerase, we chose the Gal4 DNA-binding domain in order to anchor the chimeric enzyme to a specific sequence. However, it turns out that, whereas supercoiling of the template by the fusion protein requires the presence of the Gal4 domain in the fusion protein, it does not require the presence of a Gal4 cognate site on the DNA.

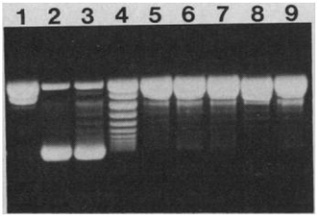

Fig. 3. Template supercoiling by Gal4-T7 RNAP. PEAO17 contains a single T7 promoter, located 523 bp downstream of a 17-bp consensus Gal4-binding site. The plasmid was constructed by inserting a 466-bp fragment of DNA derived from the Hind III to Sal I sites of pBR322, into a Kpn I site located 9 bp away from Gal4-binding site of pMH100, a derivative of pUC18 with a single 17-bp Gal4-binding site (*19*). A 38-bp fragment, Sac I to Pvu II, from pGEM-3 (Promega), which contains a T7 promoter, was inserted immediately downstream of the pBR322 DNA segment to give pEAO17. Each 80-μl reaction mixture contained 2.4 μg of template DNA, *E. coli* DNA topoisomerase I (at 12.5 μg/ml), 400 μM each of ribonucleoside triphosphates unless stated otherwise, 20 mM Hepes, pH 7.5, 7 mM $MgCl_2$, 40 mM KCl, bovine serum albumin (at 100 μg/ml), and 7 mM 2-mercaptoethanol. Ribonuclease A (12.5 μg/ml) was also included in some of the samples. Reaction mixtures were first heated at 37°C for 10 minutes. Either Gal4-T7 RNAP, or purified T7 polymerase plus purified Gal4(1–147), at the ratios indicated below, were added. After 30 minutes, the reactions were stopped, and the samples were deproteinized with proteinase K, extracted with phenol, and precipitated with alcohol. Agarose gel electrophoresis of the purified DNA samples was then carried out. (Lane 1) Relaxed pEAO17 used as the template in these experiments; (lanes 2 and 3) samples transcribed with fusion protein (22 μg/ml) in the absence and presence of RNase A, respectively; (lane 4) same as that in lane 3 but the amount of UTP during transcription was reduced to 4 μM. Samples in lanes 5 to 9 were transcribed with T7 polymerase (2.2 μg/ml), and purified Gal4(1–147) was added in a molar ratio of Gal4(1–147) to T7 polymerase of 0, 1, 5, 25, and 125, respectively.

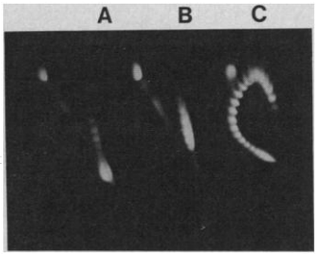

Fig. 4. Two-dimensional gel analysis of pEAO17 after transcription by Gal4-T7 RNAP (**A**) or T7 polymerase (**B**) in the presence of excess RNase A and *E. coli* topoisomerase I. (**C**) A reference distribution of pEAO17 topoisomers obtained by relaxing the supercoiled form in the presence of different amounts of ethidium. Samples were placed in wells spaced 5.3 cm apart in the horizontal direction on a slab (20 by 20 by 0.5) of 0.9 percent agarose. The gels were subjected to electrophoresis in the first dimension (top to bottom) at room temperature in 0.1 M tris base, 0.1 M sodium borate, 2 mM Na_2EDTA, pH 8.3, and chloroquine diphosphate (0.4 μg/ml). After 20 hours at 50 V, gels were soaked for 3 hours in the same buffer containing chloroquine diphosphate at 2 μg/ml. The gels were then subjected to electrophoresis in the second dimension, left to right, in fresh soaking buffer at 50 V for 12 hours. The chloroquine was removed by soaking in water, and the gels were stained with ethidium and photographed over an ultraviolet light source.

Four distinct covalently closed and relaxed DNA templates were incubated separately with the Gal4-T7 RNAP fusion protein, in the presence of *E. coli* DNA topoisomerase I and excess RNase A. After deproteinization, the DNA product was analyzed by agarose gel electrophoresis (Fig. 6). The accumulation of supercoils in the transcribed samples was evident whether the templates contained Gal4-binding sites (Fig. 6, pEAO17, lanes 1 to 3; pEAO18, lanes 7 to 9), or not (pEAO17ΔGal4, lanes 4 to 6; pEAO18ΔGal4, lanes 10 to 12). These results also show that the presence of an additional T7 promoter in pEAO18 and pEAO18ΔGal4 had little effect on template supercoiling. The lack of cryptic Gal4 cognate sequences in the ΔGal4 plasmids is supported by the fusion protein-mediated retention of pEAO17 and pEAO18 DNA, but not their ΔGal4 derivatives, to nitrocellulose filters.

Trapping of chimera-generated supercoils by nick ligation. To test further the idea that supercoiled domains are generated by

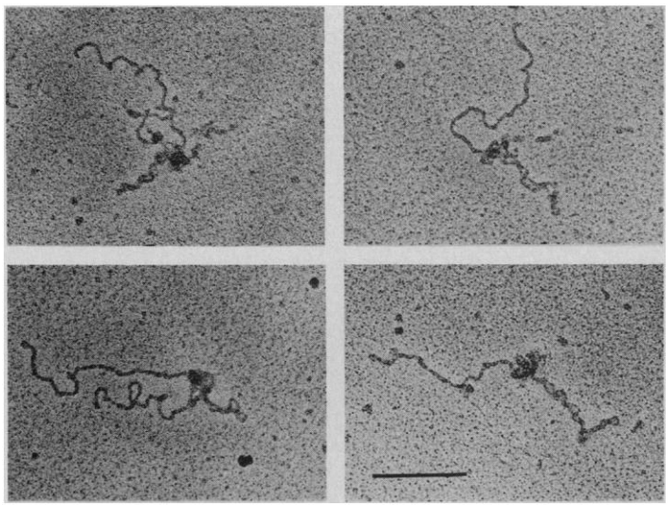

Fig. 5. Electron microscopy of linear DNA templates transcribed with Gal4-T7 RNAP fusion protein. The DNA used was Hind III–cut pEAO18, a plasmid derived from pEAO17 by the insertion of a second T7 promoter on the other side of the Gal4 cognate site. The two T7 promoters in this linearized plasmid were oppositely oriented and each one is separated by about 600 bp from the Gal4-binding site near one end. A 25-μl reaction mixture containing DNA (3 μg/ml) and Gal4-T7 RNAP (16 μg/ml) in 100 mM NaCl, 7 mM $MgCl_2$, 20 mM Hepes (pH 7.5), 7 mM 2-mercaptoethanol was incubated at 30°C for 5 minutes. A mixture containing all four nucleotide triphosphates (NTP's) was then added to give a final concentration of 400 μM for each NTP. After 5 minutes at 37°C, samples were fixed with glutaraldehyde as described previously, and 5 μl of each sample was applied to polylysine-coated carbon film on a 400-mesh grid as described (*20*). After 1 minute, excess liquid was removed by gentle aspiration. The grids were washed in 100 mM ammonium acetate and stained by touching a drop of 1 percent aqueous uranyl acetate. On rinsing with 10 mM ammonium acetate, the grids were dried and rotary shadowed with tungsten and examined at 40 kV in the electron microscope. The bar represents 160 nm.

nonspecific anchoring of Gal4-T7 RNAP, we performed a series of experiments by incubating, in the absence of *E. coli* DNA topoisomerase I, the chimeric enzyme with DNA templates containing a nick at a specific position. Supercoils in such a template can only accumulate in a domain without the nick, and they can be trapped by the ligation of the nick.

Supercoiled pEAO17, or its ΔGal4 derivative in which the Gal4 cognate site had been deleted, was first digested with Hind III in the presence of excess ethidium bromide to introduce a nick at the Hind III site (*14*); the nicked templates were banded in CsCl–ethidium bromide density gradients to remove undigested supercoiled DNA. The purified nicked DNA, which also contained a small fraction of linear DNA, was incubated first with the fusion protein in the transcription buffer containing RNase A and NAD (nicotinamide adenine dinucleotide), the cofactor for *E. coli* DNA ligase (*15*). All four ribonucleoside triphosphates were then added at 37°C to initiate transcription, and 10 minutes later *E. coli* DNA ligase was added to seal the nick in the DNA.

The relative locations of the nick, Gal4-binding site, and phage T7 promoter in Hind III-nicked pEAO17 are shown in Fig. 7. If the chimeric enzyme is anchored within the shorter arc between the nick and the promoter, positive supercoils generated by transcription could not accumulate because of the presence of the nick; thus ligation of the nick would trap the transcription-generated negative supercoils in the DNA. Conversely, if the chimeric enzyme was anchored within the longer arc between the nick and the promoter, then negative supercoils could not accumulate and transcription-generated positive supercoils would be trapped by ligation of the nick.

The electrophoretic patterns of three samples on the same two-dimensional gel were compared (Fig. 7, A to C). For the untreated DNA, most was in the nicked circular form, with a smaller amount of linear DNA (the spot diagonally downward from the nicked DNA, Fig. 7A). When ligation was carried out in the transcription mixture containing the fusion protein but in the absence of ribonucleoside triphosphates, a streak of covalently closed DNA topoisomers of different linking numbers was formed (Fig. 7B). These topoisomers represent the Boltzmann population of relaxed DNA under transcription conditions (*16*). The topoisomer distribution was strikingly different when ligation of the nick was carried out after incubation of the DNA with the fusion protein in the presence of the triphosphates (Fig. 7C). There was a faint streak of spots pointing downward from the relaxed topoisomers, and an arc of spots starting from the other end of the relaxed DNA topoisomers and going counterclockwise. The former represent topoisomers that were positively supercoiled at the time of ligation, and the latter represent topoisomers that were negatively supercoiled at the time of ligation (compare the pattern with that of a control mixture of negatively supercoiled topoisomers shown in Fig. 7E). The relative intensities of the spots show that more negative supercoils than positive supercoils were trapped by ligation of the nick in the DNA undergoing transcription. This in turn shows that the chimeric enzyme anchors preferentially in the shorter DNA segment going upstream from the promoter to the nick.

When ligation was carried out with pEAO17ΔGal4 lacking the Gal4-binding site, the distribution of topoisomers (Fig. 7D) was not significantly different from that obtained with pEAO17 (Fig. 7C). Thus, specific binding of the Gal4-binding domain to its recognition sequence is not obligatory, in agreement with the conclusion drawn earlier.

The chimera as a sequence-specific DNA supercoiling enzyme. Three different experimental approaches, namely the removal of negative supercoils by *E. coli* DNA topoisomerase I, electron microscopy, and the trapping of supercoils by ligation of a nick at a specific position, all show that the fusion of the DNA-binding domain of yeast *GAL4* gene product to the NH_2-terminus of phage T7 RNA polymerase creates a chimera that supercoils the DNA template effectively as it transcribes. Although template supercoiling by the phage polymerase itself has been reported previously, a much higher level of synthesis of long RNA is obligatory; little supercoiling is detectable at lower levels of transcription or when RNase is present to degrade the RNA as it is being synthesized (*2*).

Thus, anchoring of an RNA polymerase on its template causes the template to supercoil as transcription proceeds, as was predicted (*1, 4*). Furthermore, it appears that nonspecific DNA-protein interac-

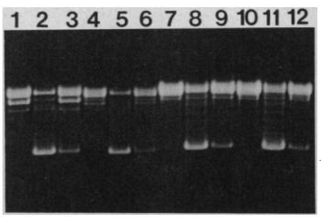

Fig. 6. Independence of template supercoiling by Gal4-RNAP on the presence of Gal4 cognate sequences. (Lane 1) Relaxed pEAO17 prior to transcription; (lanes 2 and 3) the same template after transcription with Gal4-T7 RNAP (7.5 and 1.0 μg/ml, respectively) in the presence of RNase A (25 μg/ml) and *E. coli* DNA topoisomerase I (12.5 μg/ml). The same experiment was repeated with three different template DNA's as follows. (Lanes 4 to 6) The plasmid pEAO17ΔGal4 was derived from pEAO17 by the removal of a small Hind III to Sma I fragment containing the Gal4 binding site; (lanes 7 to 9) pEAO18, a plasmid containing one Gal4-binding site and a pair of T7 promoters, each located approximately 600 bp from the Gal4 site and transcribing away from it; (lanes 10 to 12) pEAO18ΔGal4, a template derived from pEAO18 by the deletion of a 41-bp Hind III–Sma I segment containing the Gal4 cognate site.

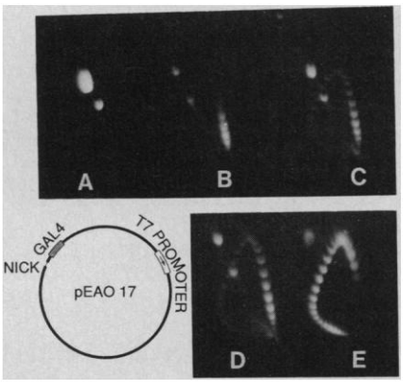

Fig. 7. Trapping of supercoils generated by the Gal4-T7 RNAP on nicked templates by *E. coli* DNA ligase. The relative positions of the nick, Gal4-binding site, and T7 promoter are indicated in the drawing shown. Portions (50 μg) of pEAO17, and a second plasmid similar to pEAO17ΔGal4, but containing a single Hind III restriction site, were digested at 26°C for 90 minutes with 60 units of Hind III in a total volume of 800 μl (in 10 mM tris, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, and ethidium bromide at 37.5 μg/ml). Residual supercoiled DNA was removed by CsCl–ethidium bromide density gradient centrifugation. (**A**) The electrophoretic pattern of purified pEAO17. (**B** and **C**) Each reaction mixture (40 μl) contained 2.5 μg of the nicked pEAO17 DNA, Gal4-T7 RNAP (28 μg/ml), RNase A (25 μg/ml), and 25 μM NAD. After a 10-minute incubation at 37°C, all four NTP's were added to the sample shown in (C) but not to the sample shown in (B), to a final concentration of 750 μM. Incubation was then continued for 10 minutes, and 5 units of *E. coli* ligase (New England Biolabs) were then added to each reaction mixture. After another 20 minutes at 37°C, reactions were terminated by the addition of SDS, EDTA, proteinase K to 1 percent, 12.5 mM, and 500 μg/ml, respectively. After digestion with proteinase K, extraction with a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1, by volume) and ethanol precipitation, samples were analyzed on two-dimensional chloroquine gels as described in the legend to Fig. 4. (**D**) Identical to (C) except that the DNA template lacks a Gal4 cognate site. (**E**) The pattern of migration of reference pEAO17 topoisomers of different linking numbers.

tions accompanying the addition of the Gal4 DNA-binding domain to the phage polymerase is sufficient to effect template supercoiling (*17*). As mentioned earlier, RNA polymerases are often in contact with regulatory factors prior to the initiation of transcription, and thus DNA template supercoiling might commence with the initiation event prior to the intervention of the DNA topoisomerases. The availability of a simple protein of the kind described above should facilitate the design of experiments on the consequences of this localized supercoiling.

Because of the sequence-specificity of T7 RNA polymerase, the Gal4-T7 RNAP chimera can be viewed as a sequence-specific DNA supercoiling enzyme. Further improvement of the sequence-specificity could possibly be achieved by the use of different DNA binding domains instead of that of Gal4, or by optimizing the separation between the promoter and the sequence that is recognized by the NH_2-terminal DNA-binding domain of the chimera. Expression of such a chimera in vivo should permit the supercoiling of a particular region of intracellular DNA, which would in turn provide an invaluable tool in assessing the physiological effects of DNA supercoiling.

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8. This segment contains an 85-bp Eco RI to Hind III fragment isolated from pE916B, a derivative of pE916A originally constructed by M. Carey and subsequently modified by M.-A. Bjornsti. This fragment carries three tandemly positioned 19-bp sequences GCGGAGTACTGTCCTCCGA, which bears a single copy of the 17-bp consensus Gal4 cognate site [E. Giniger, S. Varnum, M. Ptashne, *Cell* **40**, 767 (1985); R. Bram and R. Kornberg, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 43 (1985)].
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