



## Torsional Rigidity of Positively and Negatively Supercoiled DNA

Paul R. Selvin; David N. Cook; Ning G. Pon; William R. Bauer; Melvin P. Klein; John E. Hearst

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alent amounts of IL-2 production from the two chimeras. For both activated constructs, multiple substrates are apparent; phosphorylation activity rose rapidly over 5 min and then fell by 20 min. A reproducible difference in the substrate patterns for the two constructs was observed. Whereas several of the substrates were shared (for example, 120 and 36 kD), others are differentially phosphorylated. Tac- $\zeta$  resulted in the more prominent appearance of substrates at 140 and 65 kD (which represented the chimeric protein), whereas Tac- $\epsilon$  resulted in the prominent appearance of substrates at 100, 72 to 75, 67, and 38 kD. The 100-kD Tac- $\epsilon$  substrate comigrates with a prominent substrate seen in BW-fused T cell hybridomas and in human peripheral blood lymphocytes after stimulation of the intact TCR complex (14, 15). These differences suggest that the biochemical consequences of signaling through the  $\zeta$  and  $\epsilon$  tails are distinct. Whether this reflects the activation of different Tyr kinases must be addressed; these results raise the possibility that these two signaling-competent cytoplasmic tails of the TCR may provide distinct information to the cell.

Our data show that at least two subunits of the eight-chain TCR complex are independently capable of signal transduction, leading to both Tyr kinase activation and the production of IL-2. The ability of CD3  $\epsilon$ , in addition to  $\zeta$ , to signal resolves the dilemma raised by the findings that the cytoplasmic tail of  $\zeta$  alone can mimic TCR-mediated activation (8–10), yet partial TCR complexes lacking any  $\zeta$  chain can activate hybridoma cells to produce IL-2 (11). The presence of two proteins in a single receptor complex independently able to transduce signals raises several questions about the functioning of the intact receptor. Are the cytoplasmic tails of  $\zeta$  and CD3  $\epsilon$  redundant? Do they cooperate in the activation of the same signaling pathways? Do they couple to similar but distinct signaling pathways? Some support for the last possibility is suggested by the difference in substrate phosphorylation patterns produced by the Tac- $\zeta$  and Tac- $\epsilon$  chimeras. Thus, the multichain TCR complex may not generate signals through a single subunit. The 22-amino acid functional motif of CD3  $\epsilon$  described here can be aligned to a consensus sequence present in the COOH-terminal domains of subunits of various receptors (16). Whether this represents an identical coupling motif or variations on a general theme, perhaps for preferential coupling to different Src family kinases, can now be addressed.

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## Torsional Rigidity of Positively and Negatively Supercoiled DNA

PAUL R. SELVIN,\* DAVID N. COOK, NING G. PON, WILLIAM R. BAUER, MELVIN P. KLEIN, JOHN E. HEARST

**Time-correlated single-photon counting of intercalated ethidium bromide was used to measure the torsion constants of positively supercoiled, relaxed, and negatively supercoiled pBR322 DNA, which range in superhelix density from +0.042 to -0.123. DNA behaves as coupled, nonlinear torsional pendulums under superhelical stress, and the anharmonic term in the Hamiltonian is approximately 15 percent for root-mean-square fluctuations in twist at room temperature. At the level of secondary structure, positively supercoiled DNA is significantly more flexible than negatively supercoiled DNA. These results exclude certain models that account for differential binding affinity of proteins to positively and negatively supercoiled DNA.**

**T**HE MOTIVATION FOR STUDYING the torsional rigidity of supercoiled DNA is threefold. First, such study provides a stringent test of current theoretical models of DNA that assume that the twisting (and bending) internal forces are harmonic and isotropic (1, 2). Second, the twisting rigidity of DNA is an important determinant of the energetics of formation of complicated nucleoprotein structures (3). Third, although the torsion constant has been extensively studied for linear DNA (4–8), its dependence on superhelicity under physiological conditions has not been systematically measured.

We obtained torsion constants by fitting the fluorescence anisotropy decay of ethidium bromide intercalated in various supercoiled topoisomers of pBR322 to the theory of Barkley and Zimm with the use of a Marquardt-based algorithm (2, 9). The anisotropy decay arises from the reorientation of excited-state ethidium bromide due to the twisting (and bending) of the DNA helix, and it was detected by time-correlated single-photon counting. The Barkley-Zimm theory, which models DNA as a uniform elastic rod that bends and twists in a viscous medium, is a harmonic approximation. The fitted value of the torsion constant depends weakly on the bending rigidity chosen, which we assume is constant and independent of superhelicity. Because of the weak dependence on bending and the fact that DNA is not severely bent in a plectonemic superhelix (10), this assumption is justified.

We chose a bending constant that yields a persistence length of 525 Å. We also analyzed the data with the exclusion of the effect of bending' (infinite bending constant), which yields a lower limit to the torsion constant. In this limit, the Allison-Schurr and Barkley-Zimm models of DNA dynam-

P. R. Selvin, Department of Physics, University of California, Berkeley, and Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, Berkeley, CA 94720.

D. N. Cook, N. G. Pon, J. E. Hearst, Department of Chemistry, University of California, Berkeley, and Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, Berkeley, CA 94720.

W. R. Bauer, Department of Microbiology, School of Medicine, State University of New York, Stony Brook, NY 11794.

M. P. Klein, Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, Berkeley, CA 94720.

\*To whom correspondence should be addressed; present address: Laboratory of Chemical Biodynamics.

ics are mathematically identical. The fractional difference in torsion constants between topoisomer samples is nearly independent of the particular bending rigidity chosen. The torsion constant increases by approximately 1.6 when bending is included in the analysis.

The parallel, perpendicular, and anisotropy curves for a negatively supercoiled sample are shown in Fig. 1. The quality of the data is representative of all samples. A two-exponential fit to magic angle data, not shown, deconvoluted with the instrument response function, yields  $\chi^2 < 1.3$  and shows that >99% of the fluorescent photons arise from intercalated ethidium bromide (lifetime  $\approx 22.5$  ns) with the remaining <1% arising from free dye (lifetime  $\approx 1.6$  ns). This is true for all samples, including positively supercoiled DNA. The anisotropy fit to each sample, derived with the use of just a single torsion constant (rather than a sum) is excellent ( $\chi^2 < 1.3$ ), which indicates that the harmonic approximation is good for each narrow distribution of topoisomers.

If the torsion constant changes between samples that differ significantly in average superhelicity, then the twisting potential has an anharmonic component. To determine the sensitivity of our system to changes in torsion constant, we measured the anisotropy

decay of phage  $\lambda$  DNA in sucrose solutions with viscosities from 1.0 to 2.45 cP (ranging from 0 to 24% sucrose final concentration). According to theory, the anisotropy decay depends on the inverse square root of the product of the viscosity and torsional constant (1, 2). Therefore, varying the viscosity  $\eta$  should simulate a change in the torsion constant, and a plot of  $(1/\eta)^{1/2}$  versus the anisotropy fit parameter should yield a straight line. Figure 2 shows that fluorescence polarization anisotropy is sensitive to changes in viscosity, and hence torsion constant, of 10% or less (11).

The best fit torsion constants for our topoisomer samples under physiological salt and low-salt conditions are shown in Fig. 3. At 175 mM ionic strength, the torsion constant increases linearly as the superhelix density becomes more negative (Fig. 3). At low ionic strength ( $\sim 7.5$  mM) and for nonnegative superhelix densities, the same trend is observed (Fig. 3).

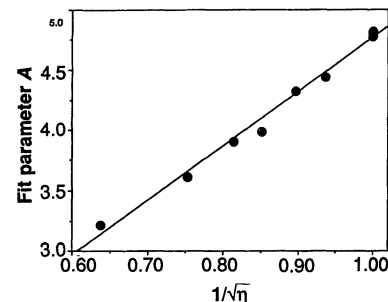
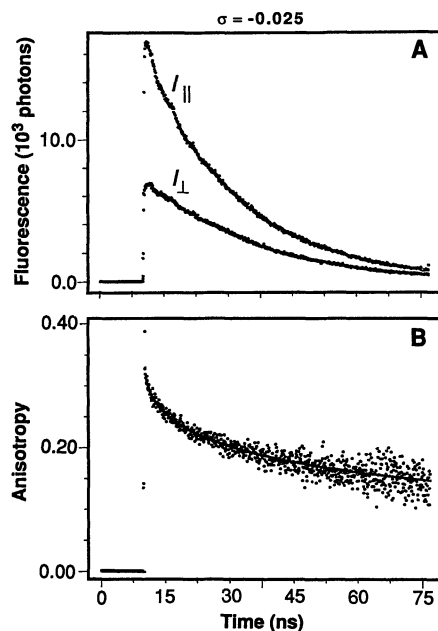
Two other groups have studied the dependence of the torsion constant on superhelicity using fluorescence depolarization. Millar *et al.* (7) compared pBR322 of "native" superhelix density to linear calf thymus DNA in 0.2 M salt. They found that negatively supercoiled pBR322 is torsionally more stiff than calf thymus DNA. Schurr and co-workers have studied supercoiled

DNA under a variety of conditions (12). In one study with 0.1 M salt, they concluded that there is no variation in torsional stiffness between three samples at superhelix densities of 0,  $-0.048$ , and  $-0.083$  (13). In a later study with low-salt conditions ( $\sim 15$  mM), they concluded that the torsion constant increases with negative supercoiling in a complex manner that varied over many weeks (11).

The torsion constant of our relaxed sample,  $1.9 (\pm 0.1) \times 10^{-19}$  erg-cm, is in good agreement with the fluorescence anisotropy work of others (6, 7, 14), although there is some variability in the literature depending on assumptions of helix radius, persistence length, and contribution of bending to depolarization. Cyclization studies, which measure the probability of ring closure of short ( $\leq 500$  bp) DNAs (4, 8), generally yield somewhat higher values for the torsion constant, although Taylor and Hagerman have recently measured a value of  $2.0 (\pm 0.2) \times 10^{-19}$  erg-cm using this method (5, 15). This method, however, cannot be used to study topoisomers outside the thermally accessible states near the relaxed topoisomer.

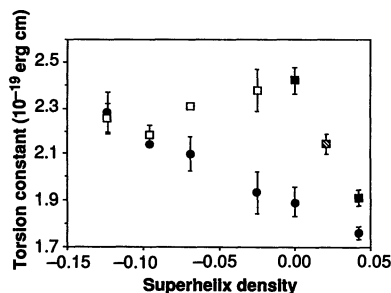
One possible complication with our measurement is that highly negative supercoiling can induce structural transitions in DNA. For our physiological salt conditions, we argue that any secondary structures in non-B form that may arise from superhelical stress, such as left-handed Z-DNA or cruciforms, would alter the measured torsion constant only by a small amount. Although cruciforms are extruded in pBR322 at superhelix densities more negative than  $-0.08$

**Fig. 1. (A)** Parallel and perpendicular and **(B)** anisotropy decay modes of the fluorescence of intercalated ethidium bromide in negatively supercoiled DNA ( $I$ , intensity). The quality of the data is indicative of all of the topoisomers measured. Conditions: DNA (200  $\mu\text{g/ml}$ ) and 2  $\mu\text{M}$  ethidium bromide buffered at pH 8.0 in tris. Physiological salt solution contained 50 mM tris buffer, 150 mM KCl, and 1 mM EDTA; low-salt solution contained 5 mM tris and 0.5 mM EDTA. We prepared positively supercoiled pBR322 in vivo by treating strain AS19 (pBR322) with novobiocin according to the protocol of Lockshon and Morris (25), and the sample is thus a kinetic distribution of topoisomers. Our estimate of  $\sigma = +0.042$  is a minimum estimate based on the ability of agarose gel electrophoresis to resolve overwound topoisomers. Negatively supercoiled samples were prepared by the method of Singleton and Wells (26). A reference topoisomer distribution, which was used to define the relaxed linking number  $Lk_0$ , was prepared in a physiological salt buffer identical to that used for the spectroscopy. Gel electrophoresis of samples after exposure to light showed no light-induced nicking of the DNA. Approximately 5% of the DNA was nicked before spectroscopy, presumably because of handling. The solid line in the anisotropy curve is the Barkley-Zimm best fit curve, derived with the use of a nonlinear Marquardt-based fitting algorithm. The dipole moment of the ethidium with respect to the helix axis was assumed to be  $70^\circ$  (27), and the helix radius was assumed to be 13.4  $\text{\AA}$  (7). The anisotropy function is the standard  $(I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})$ , corrected for laser light fluctuations and polarization sensitivity of the detection system. The time resolution of our instrument was 75 ps, and measurements were taken from 0 to 75 ns, the period in which twisting dominates the fluorescence depolarization. The instrument response function (full-width at half maximum  $\leq 75$  ps) was deconvoluted only for the magic angle data. Interference filters were used to rigorously exclude Raman and Rayleigh scattered light, and excitation was effected with a mode-locked dye laser set at 550 nm to minimize fluorescence from free dye (28).



**Fig. 2.** Dependence of anisotropy decay on relative viscosity. Fits were made to the anisotropy decay of phage  $\lambda$  DNA by allowing both the initial anisotropy and a fit parameter  $A$  to vary. According to theory (1, 2),  $A = 2k_B T / (\pi B \sqrt{\eta C})$ , where  $B$  is the hydrated DNA radius (13.4  $\text{\AA}$ ) and  $\eta$  is the relative viscosity. Therefore, a change in viscosity simulates a change in the torsion constant. Plotting  $A$  versus  $1/\sqrt{\eta}$  should yield a straight line. The data agree with theory. The sucrose concentrations were measured to four significant figures with a Bausch and Lomb refractometer. The viscosity as a function of sucrose concentration and temperature is extensively tabulated in (29).

**Fig. 3.** Torsion constants of topoisomer samples in 150 mM KCl, 50 mM tris, 1 mM EDTA (●); ionic strength  $\approx 175$  mM) or in 5 mM tris and 0.5 mM Na<sub>4</sub> EDTA (■, □, and crosshatched square; ionic strength 7.5 mM), all at pH 8.0. Samples were prepared as described in the legend to Fig. 1. Error bars represent the SD when the number of measurements  $n \geq 3$  or the spread between independent measurements when  $n = 2$ . When error bars are absent, the deviation is less than the symbol width. At high ionic strength, the  $\sigma = 0$  data is the average of measurements made on linear and relaxed plasmid DNA. At high ionic strength the torsion constant increases linearly with negative supercoiling. For the low-salt data, closed squares (■) represent topoisomers where alternative structures are not likely to exist; the hatched square represents a linearized DNA sample; open squares (□) represent topoisomers under moderate to high superhelical stress where alternative structures may complicate the analysis (see text). Both low- and high-salt data were analyzed with the same persistence length and helix radius, because there are uncertainties in the literature about the exact values at low ionic strength for the helix radius, persistence length, and Poisson's ratio. Therefore, a direct comparison of the torsion constants between the different ionic strengths is not appropriate. However, it is clear that positively supercoiled DNA is torsionally more flexible than relaxed or negatively supercoiled DNA. The low-salt data were obtained by precipitating the high-salt samples and resuspending in the low-salt buffer. Consequently, the actual superhelix density of the low-salt samples is more negative than that shown (30). (There is no reliable measurement in the literature on the magnitude of the shift at this ionic strength.) The linear DNA sample, which is free to assume an equilibrium twist equivalent to the low-salt reference state, is therefore presented as a positively supercoiled ( $\sigma = 0.02$ ) sample with this convention.



(16, 17), they constitute only a small fraction of the DNA, on the order of 25 bp out of the total 4363 bp. Furthermore, pBR322 does not contain extensive alternating purine-pyrimidine tracts, and the formation of Z-tracts is strongly dependent on length. For instance, Singleton *et al.* (17) find that Z-DNA forms at a superhelicity of  $-0.072$  in pBR322 at 200 mM ionic strength only if a 26-bp or longer alternating G-C tract is inserted into the plasmid (18).

Under physiological salt conditions, alternative structures would, in the worst case, lead to an underestimation of the true torsion constant. Two possibilities can be considered. (i) If the alternative structure is

more stiff than B-DNA, its presence will not alter the dynamics, and hence the torsional rigidity, reported by the ethidium (1), because a localized stiff spot in a flexible rod does not significantly alter the overall dynamics. (ii) If the alternative structure is more flexible, it can reduce the measured torsional rigidity—local weakness can alter the dynamics of an otherwise stiff rod (1). In this case, the increase in torsion constant found with increasing negative superhelicity is a lower bound for the rigidity of the remaining B-form DNA. Our assumption of no alternative structures, therefore, yields a lower limit to the nonlinearity of the DNA.

Our interpretation is a lower limit for another reason: cruciforms and Z-tracts, if present, remove superhelical turns that would make negatively supercoiled DNA less torsionally stressed, and, in effect, they contract the  $x$ -axis of Fig. 3. The slope would then be steeper, indicating that the DNA is torsionally even more nonlinear than shown in Fig. 3.

At low ionic strength, positively supercoiled DNA is torsionally more flexible than relaxed DNA. At nonnegative superhelix densities, the torsion constant also increases more rapidly with the change in linking number ( $\Delta Lk$ ) than it does under physiological ionic strength (closed squares, Fig. 3). This is in part because a greater fraction of  $\Delta Lk$  goes into the change in twist ( $\Delta Tw$ ) at low salt concentration—the superhelix radius increases (10, 19) and makes the writhe ( $Wr$ ) more energetically costly. Hence, the base pairs sample a greater range in equilibrium twist angle and make the nonlinearity in torsion constant more evident. Furthermore, the increased superhelix radius at low salt concentration leads to greater torsional stress than that at high salt concentration. The greater torsional stress under these conditions

may lead to significant alternative structures at negative superhelix densities, making the interpretation of these data problematic (open squares, Fig. 3).

On the basis of the above arguments, our data indicate that the torsion constant for DNA linearly increases with superhelicity. If the DNA is modeled as a series of base pairs (flat disks) coupled by torsional springs, this linear increase in torsion constant indicates that a cubic term should be added to the twisting Hamiltonian (20). At physiological ionic strength, the best fit line of torsion constant per base pair versus  $\sigma$  is:

$$C/h = m\sigma + b$$

where  $C$  is the torsion constant,  $h$  is the height per base pair ( $=3.4 \times 10^{-8}$  cm),  $m$  is the experimentally determined slope,  $\sigma$  is the fractional change in linking number ( $\Delta Lk/Lk_0$ ) and  $b$  is the  $y$  intercept, which is also the torsion constant per base pair in the absence of topological stress. We find that  $b = 135$  and  $m = -218$  (both in units of  $k_B T$  per base pair, where  $k_B T$ , Boltzmann's constant times temperature, is the thermal energy). Note that this approximation must break down at sufficiently positive superhelical density, or a zero or negative torsion constant would result.

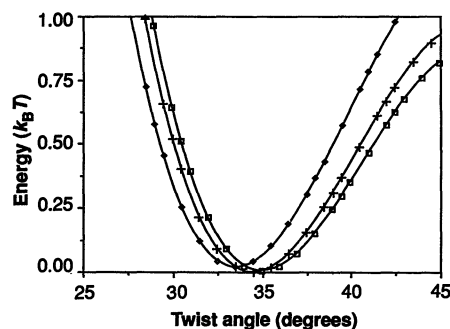
To understand in molecular terms the change in torsion constant after supercoiling, we must know what fraction  $F$  of  $\Delta Lk$  goes into  $\Delta Tw$  and into  $Wr$ . ( $F \equiv \Delta Tw/\Delta Lk$ ; recall that  $\Delta Lk = \Delta Tw + Wr$ ). The change in twist per base pair is simply  $\Delta Tw/N$ , or  $F\Delta Lk/N$ , where  $N$  is the number of base pairs in the DNA molecule.

Three groups have estimated  $F$ . Klenin *et al.*, using Monte Carlo simulations, found that  $F \approx 0.3$  (0.2 to 0.4) (21). Boles *et al.*, using electron microscopy and topological methods, found that  $F \approx 0.28$  (22). Hunt and Hearst (19), using analytical mechanics, estimated that  $F \approx 0.2$  to 0.3 for physiological superhelix densities. In fact,  $F$  is most likely not a constant, but instead a function of superhelicity (19). For the purposes of our discussion, we assume  $F$  is a constant.

The twisting potential energy per base pair,  $U$ , of a supercoiled DNA molecule can now be written:

$$U \approx \frac{b}{2} (\phi_0' - \phi_0)^2 + \frac{Fm}{3} (\phi_0' - \phi_0)^3 + \frac{b'}{2} (\phi - \phi_0')^2 + \frac{Fm}{3} (\phi - \phi_0')^3$$

where  $\phi_0$  is the equilibrium angle between base pairs for relaxed DNA (0.601 radians or 34.45°),  $\phi_0'$  is the equilibrium angle for a particular topoisomer ( $\phi_0' = 2\pi F \Delta Lk/N + 0.601$ ),  $b'$  is the torsion constant at the superhelix density corresponding to equilibrium twist angle  $\phi_0'$  (equal to  $C/h$  in the previous



**Fig. 4.** Plot of the potential energy surface for base pair twisting at superhelix densities of  $\sigma = 0.00$  (+, 34.45°),  $-0.084$  (◆, 33.49°), and  $+0.042$  (□, 34.93°), assuming that  $\Delta Tw = 0.33 \Delta Lk$ . This plot is based on an extrapolation to large deviations in twist angle of the linear relationship of the torsion constant versus superhelix density data in Fig. 3. Such an extrapolation has clear limits, because, at extreme positive deviations in twist angle, the torsion constant is predicted to go to zero. However, the equilibrium twist angle,  $\phi_0'$ , varies only slightly with  $\Delta Lk$  when the partition factor  $F = 0.33$ , as we have assumed (19, 21, 22).

equation), and  $\phi$  is the instantaneous angle between neighboring base pairs for the particular conformation. The first two terms are the energy required to change the equilibrium twist angle from  $\phi_0$  to  $\phi_0'$ , and the last two terms represent the energy arising from fluctuations about the new equilibrium angle. The twisting potential energy as a function of twist angle between base pairs is shown in Fig. 4 for  $F = 0.33$ .

The magnitude of the anharmonicity for the relaxed molecule can be estimated by determining the size of the cubic term relative to the quadratic term in the potential energy expression when  $(\phi - \phi_0') \approx 5^\circ$ , the root-mean-square deviation due to thermal energy. For  $F = 0.33$ , the correction term represents a change of  $\sim 15\%$  in the height of the potential energy surface for the relaxed topoisomer at  $U = 1/2 k_B T$ . This value is sufficiently small that the perturbation-type expansion we have used is expected to be valid. Moreover, the Barkley-Zimm model, a harmonic approximation, is reasonable to use in analyzing each narrow topoisomer distribution, as we have done.

Our results have consequences for the formation of protein complexes on supercoiled DNA. The araC protein forms looped structures on negatively supercoiled DNA, but not on relaxed minicircle DNA (23). Similarly, the lac repressor can form stable loops on negatively but not positively supercoiled minicircle DNA when a twist is required to bring protein binding sites onto the same side of the helix (24). Our results imply that the preference for negatively supercoiled DNA is not due to increased twisting flexibility of the negatively supercoiled DNA. Rather, we speculate that these DNA-looping proteins may instead be sensitive to the spatial orientation of juxtaposed DNA strands, which changes for supercoils of opposite sign.

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- The absolute value of the torsion constant is not of

- primary interest here, because it is changes in torsion constant between topoisomers that indicate relative stiffness and nonlinearities in the twisting potential. Moreover, there is no a priori reason why cyclization and fluorescence should yield the same absolute numbers, because the cyclization method measures changes in net twist on the millisecond to second time scale, whereas the fluorescence measures fluctuations in twist on the nanosecond time scale. It is well known in polymer theory that measured stiffness can be a function of frequency and net strain, either because of nonlinearities or resonances in the molecule.
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- Even at superhelix densities as high as  $-0.12$ , a conservative analysis of Singleton's data (17) indicates that, at most, 1% of the sequence in pBR322 exists in alternative structures. At  $\sigma = -0.12$  and for a plasmid of 4400 bp, a B- to Z-DNA transition involving only 40 bp would be sufficient to make the effective superhelix density  $-0.08$ . The data of Singleton *et al.* (compare with figure 1c, plasmid pRW451) demonstrate that there are no S1 nuclease-sensitive sites in pBR322 at this level of supercoiling.
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- because a detailed interpretation of their results is complicated by the high ratios of ethidium per base pair that they used to titrate supercoils.
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## Interaction of p107 with Cyclin A Independent of Complex Formation with Viral Oncoproteins

MARK E. EWEN,\* BARBARA FAHA, ED HARLOW, DAVID M. LIVINGSTON

The p107 protein and the retinoblastoma protein (RB) both bind specifically to two viral oncoproteins, the SV40 T antigen (T) and adenoviral protein E1A (E1A). Like RB, p107 contains a segment (the pocket) that, alone, can bind specifically to T, E1A, and multiple cellular proteins. Cyclin A bound to the p107 pocket, but not the RB pocket. Although both pockets contain two, related collinear subsegments (A and B), the unique sequence in the p107 pocket that occupies the space between A and B is required for the interaction with cyclin A.

THE CELLULAR PROTEIN, p107, shares a number of properties with the retinoblastoma product (RB), a known tumor suppressor. Both proteins form complexes with SV40 large T antigen (T) and the adenoviral protein E1A (1-3), and RB interacts with the human papilloma virus E7 protein (4). Complex formation may, in part, underlie the transforming functions of these viral proteins (2-6). RB also interacts specifically with several cellular proteins in vitro (13-15). These interactions may account for the proposed regulation of the cell cycle by RB (7-12). RB and cyclin A, another protein that functions to regulate the cell cycle, both

bind specifically to the transcription factor, E2F (16-21). These interactions may also contribute to cell cycle control.

The p107 protein has clear sequence similarity to RB. The RB sequence contains a stretch of  $\sim 400$  amino acids (the pocket) that can alone bind T, E1A, and the aforementioned cellular proteins (13, 22-24). The pocket is composed of two subsegments (A and B) that are separated by a spacer of  $\sim 75$  residues (Fig. 1). Foreign sequences can be substituted for the spacer without disturbing binding of T and E1A (22, 23). Similarly, a segment of p107 containing  $\sim 600$  residues (residues 252 to 816) can also bind T and E1A. The segment composed of two subsegments similar to A and B of RB. Parts A and B of p107 are separated by a spacer of  $\sim 200$  amino acids (Fig. 1) (1). Substituting the RB spacer for the spacer in p107 did not affect binding of T or E1A (1), suggesting that the

M. E. Ewen and D. M. Livingston, Dana-Farber Cancer Institute, Boston, MA 02115.  
B. Faha and E. Harlow, Massachusetts General Hospital Cancer Center, Charlestown, MA 02129.

\*To whom correspondence should be addressed.