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Specificity of the Weak Binding between the Phage SPO1 Transcription-Inhibitory Protein, TF1, and SPO1 DNA†

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ABSTRACT: The interaction of the phage SPO1 protein, transcription factor 1 (TF1), with DNA has been analyzed by membrane filter binding and by sedimentation methods. Substantially specific binding of TF1 to helical SPO1 DNA can be demonstrated by nitrocellulose filter-binding assays at relatively low ionic strength (0.08). However, TF1–DNA complexes dissociate and reequilibrate relatively rapidly and this makes filter-binding assays unsuitable for quantitative measurements of binding equilibra. Accordingly, the sedimentation properties of TF1–DNA complexes have been explored and a short-column centrifugation assay has been elaborated for quantitative measurements. Preferential binding of TF1 to the hydroxymethyluracil-containing SPO1 DNA has also been demonstrated by short-column centrifugation. TF1 binds relatively weakly and somewhat cooperatively to SPO1 DNA at many sites; TF1–DNA complexes dissociate and reequilibrate rapidly. At 20 °C in 0.01 M phosphate, pH 7.5, 0.15 KCl, one molecule of TF1 can bind to approximately every 60 nucleotide pairs of SPO1 DNA.

The SPO1 DNA-binding protein, transcription factor 1 (TF1), is synthesized after SPO1 infection of *Bacillus subtilis* 168M (Wilson and Geiduschek, 1969; Johnson and Geiduschek, 1972). Within 20 min after infection at 37 °C, more than 10^5 molecules have been synthesized in each infected cell (Johnson and Geiduschek, 1972). TF1 selectively inhibits in vitro transcription of native, bihelical SPO1 DNA (and other hmU-containing phage DNA) by bacterial DNA-dependent RNA polymerase (Wilson and Geiduschek, 1969). We have previously described the purification of this protein and some of its molecular properties (Johnson and Geiduschek, 1972; Johnson and Geiduschek, 1974).

In the experiments that we present here, we have explored the interaction of TF1 with DNA. For our first experiments along these lines, we made use of the ability of nitrocellulose membranes to bind proteins and protein–nucleic acid complexes, as first described by Jones and Berg (1966). The properties of these complexes first suggested to us that TF1 and SPO1 DNA must dissociate more readily than do complexes between DNA and RNA polymerase, repressors, unwinding proteins, or histones (Hinkle and Chamberlin, 1970; Gilbert and Muller-Hill, 1967; Riggs et al., 1970; Ptashne, 1967; Alberts and Frey, 1970; Alberts et al., 1972; von Hippel and McGhee, 1972). Accordingly, we examined TF1–DNA complexes and their dissociation by centrifugation analysis. In this paper, we present the results of these explorations and of a more detailed measurement of the equilibrium constant for DNA–TF1 binding.

Materials and Methods

(*1*) *Buffers.* The following buffers are referred to in the text by abbreviation: G: 10 mM glycine (Na), pH 10, 0.1 mM EDTA, 0.1 mM dithiothreitol. GD: buffer G but with 1.1 mM dithiothreitol. PK: 0.01 M phosphate (Na), pH 7.5, 0.15 M KCl. TM: 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$. TCED: 10 mM Tris-cacodylate, pH 6; 0.1 mM EDTA, 0.1 mM dithiothreitol (10 mM Tris-cacodylate is 10 mM Tris base, 10 mM cacodylic acid, adjusted to pH 6 with HCl). TCEDK: TCED with 0.25 M KCl. TED: 100 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM dithiothreitol. TKE: 100 mM Tris-HCl, pH 7.5, 0.1 M KCl, 0.1 mM EDTA. TKED: TKE with 0.1 mM dithiothreitol.

(*2*) *Unlabeled, ^3H- and ^{35}S-labeled TF1.* TF1 was purified through the phosphocellulose column purification step of our previously described purification method (Johnson and Geiduschek, 1972). Only peak phosphocellulose fractions of constant specific activity (units of TF1/mg of protein) or constant specific radioactivity (units of TF1/cpm) were used in these studies. The molecular-weight homogeneity of TF1 was verified by acrylamide gel electrophoresis (Laemmli,

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[1] Abbreviations used are: DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

1970). [^{3}H]TF1 gave one peak on isoelectric focusing (Johnson and Geiduschek, 1972).

For a subsequent preparation of [^{35}S]TF1, we used a slightly different source material, SPO1 *sus* F4-infected *B. subtilis* 168M, and a slightly altered purification. SPO1 *sus*F4 (gene 34) is a maturation-defective mutant; nonpermissive *B. subtilis* 168M infected with this mutant do not lyse and continue to accumulate extractable TF1 activity at late times after infection.

B. subtilis were grown from spores in a synthetic medium (Donnellan et al., 1964) with low sulfate (40 μM Na_2SO_4). When A_{500} reached 1.0 (2.4×10^8 cells/ml), 2×10^{12} infectious centers of SPO1 *sus* F4 were added per liter of culture. Twenty liters of unlabeled cells were collected and mixed with 1 L of labeled cells which had been supplemented with 35 mCi of $Na_2{}^{35}SO_4$ at infection (20 mCi of ^{35}S label had been incorporated into hot 12.5% Cl_3CCOOH-precipitable material at 60 min after infection).

The [^{35}S]TF1 was purified from these cells through the SE-Sephadex step of the previously described procedure (Johnson and Geiduschek, 1972). TF1 eluting at this step is the major protein incorporating amino acid label after phage infection. However, as the cpm/A_{235} ratio was not constant across the TF1 peak in this preparation, we used DEAE-cellulose chromatography at pH 10 to purify the [^{35}S]TF1 to homogeneity. The ^{35}S-labeled TF1 peak fractions from SE Sephadex (containing 4.8 mg of [^{35}S]TF1) were pooled and dialyzed into buffer G overnight at 4 °C. Buffer G was prepared from boiled water, and reasonable precautions were used to protect the buffer and the DEAE-cellulose column from atmospheric CO_2 contamination. The dialyzed fractions were applied to a 10-mL DEAE-cellulose column (in a disposable 10-mL syringe) in buffer G. TF1 is retained by this column beyond the void volume and elutes slowly, but does not require salt elution. Peak TF1 fractions were stored frozen at −20 °C after adding 1 mM dithiothreitol (buffer GD).

(*3*) *Labeled DNA.* To prepare ^{14}C-labeled SPO1 DNA, cells were labeled with adenine after phage infection. We obtained SPO1 DNA with a specific activity of 2000 cpm/μg from the purified phage.

To prepare ^{32}P-labeled SPO1 DNA, *B. subtilis* 168M were grown in a low-phosphate medium and infected with 10 SPO1 phages/bacterium. Ten minutes later, 0.8 mCi of carrier-free $^{32}PO_4$ (Na) was added. Lysis occurred approximately 70 min after infection. We obtained SPO1 DNA with a specific activity of 4600 cpm/μg from the purified phage.

DNA was extracted from purified phage with phenol (Mandell and Hershey, 1960). T7 DNA was labeled and purified according to Richardson et al. (1964) and T4 DNA according to Cascino et al. (1971).

(*4*) *Sedimentation Analysis of SPO1 and ϕ1 DNA.* The sedimentation rate of labeled DNA was tested in neutral (0.01 M Tris-HCl, pH 7.5) and alkaline (0.1 M NaOH) 5–20% sucrose gradients containing 1.0 M Na^+, 15 mM EDTA, and 0.1% sarkosyl (cf. Levner and Cozzarelli, 1972). Centrifugation was in a swinging-bucket rotor at approximately 200 000*g* for 55 min at 15 °C. The sedimentation distance of labeled ϕ1 and SPO1 DNAs in the neutral solvent was virtually identical with that of T4 DNA. However, Parker and Eiserling (personal communication) have recently shown that SPO1 DNA is smaller than T4 DNA and that its sedimentation constant ($S_{20,w}$) is smaller than that of T4 DNA. All preparations of SPO1 DNA contained some single-strand breaks (or alkali-labile bonds), as judged by the following criterion. In 0.1 M NaOH–sucrose gradients, the sedimentation rate of at least one-half, but never all, of the DNA was 20% greater than in neutral sucrose gradients. The remainder of the alkali-denatured SPO1 DNA was rapidly, but heterogeneously, sedimenting (>35 S) material.

(*5*) *TF1 and DNA Concentrations.* TF1 transcription-inhibition activity was determined as previously described (Johnson and Geiduschek, 1974). TF1 concentration was estimated using the Folin reaction (Lowry et al., 1951) standardized against bovine serum albumin (Armour, fraction V). We have previously reported that 1 mg of Folin equivalent corresponds to 6300 nmol of TF1 amino acid, or 0.68 mg of TF1 protein (Johnson and Geiduschek, 1972). For filter-binding experiments, we used homogeneous TF1 preparations isolated through the phosphocellulose column fractionation step (Johnson and Geiduschek, 1972), which had a specific activity of 220 units/mg of TF1 protein. ^{3}H-labeled TF1 was also isolated through the phosphocellulose column purification step and had the same specific activity. For unlabeled and ^{3}H-labeled TF1, 1 unit of transcription inhibitory activity therefore corresponds to 4.5 μg of TF1 protein.

Peak [^{35}S]TF1 fractions isolated by DEAE-cellulose chromatography at pH 10 had a specific activity of 285 units/mg of TF1 protein. This specific activity is equal to that obtained when homogeneous, phosphocellulose-purified TF1 (220 units/mg of TF1 protein) is passed in a low ionic strength phosphate buffer through Sephadex G-200 (Johnson and Geiduschek, 1974). Our preparation of [^{35}S]TF1 had a specific radioactivity of 4.9×10^7 cpm/mg of TF1 protein. The concentration of TF1 has also been determined from its absorbance at 210 or 235 nm. A specific activity of 220 units/mg of TF1 corresponds to 17 units/A_{210} and 200 units/A_{235} (A is the quantity of TF1 which gives unit absorbance at the specified wavelength when dissolved in 1 ml of buffer TCEDK).

DNA concentrations were determined from absorbance measurements at 260 nm using an extinction coefficient of 2×10^4 cm^2 g^{-1}.

(*6*) *Membrane Filter Binding.* The standard incubation volume for membrane filter-binding assays was 0.25 mL and the standard solvent was TED buffer with 10 μg/mL bovine serum albumin. 0.69 μg of ^{14}C-labeled DNA (final concentration 2.75 μg/mL) was diluted to a total of 0.225 mL and equilibrated at 30 °C. Twenty-five microliters of TF1 solution (in 0.01 M Tris-HCl, pH 7.5, 100 μg/mL bovine serum albumin, Armour, crystalline) was added to the DNA to constitute the final volume and final buffer composition, and the incubation was continued for 2 min at 30 °C. This 0.25-mL incubation mixture was then diluted with 10 mL of ice-cold TED buffer and immediately vacuum filtered through a 26-mm Schleicher and Schuell, Type B-6, nitrocellulose membrane filter, which had been presoaked for at least 10 min in TED buffer. Membranes were not further rinsed after filtration. The filtration was carried out in a stainless-steel filter holder, with a liquid capacity of approximately 15 mL. The assembly was precooled to 0 °C in ice water immediately prior to filtration. Although filtration was carried out in a room temperature environment, the samples remained at less than 7 °C for the duration of the filtration (usually 90 s). After partial drying with vacuum, the filters were processed as described below for radioactive counting.

(*7*) *Sucrose Sedimentation of TF1–DNA Complexes.* Centrifugation of linear 5–20% sucrose density gradients was carried out at 20 °C in either the SW 50.1 or SW 65 Beckman swinging-bucket rotors in nitrocellulose tubes of 5.2-mL capacity. The gradient solvent was TKE or TKED. Tubes were fractionated from the gradient bottom. Two types of gradient

centrifugation were performed. In all cases, a 0.2-mL sample containing DNA, or DNA and TF1, was layered on the gradient immediately prior to centrifugation. For gradients containing TF1, the TF1 was diluted to the desired final concentration in the appropriate solvent with 5 and 20% (w/v) sucrose, and linear 5–20% sucrose gradients were made with these stock sucrose solutions containing TF1.

(8) Short-Column Centrifugation. The sedimentation method described next was used to measure formation of relatively labile complexes between relatively rapidly and slowly sedimenting components. A somewhat different boundary sedimentation method was independently worked out by D. E. Jensen and P. H. von Hippel (1976, submitted for publication).

The following method has been used to measure binding of TF1 to DNA. A 0.40-mL sample of TF1 that has been incubated with DNA is centrifuged in a 5-mL stainless-steel tube to sediment the DNA away from unbound TF1. We determine the TF1 binding to DNA by either of two methods: (a) in the sucrose-cushion method, the sedimented TF1–DNA complex is recovered from a 0.10-mL sucrose cushion placed under the sample prior to centrifugation; (b) in the supernatant method, the disappearance of TF1 from the supernatant due to binding and sedimentation with DNA is measured. The details which follow mainly have to do with two properties of the system, which complicate the analysis: TF1 adheres to surfaces; under certain conditions, DNA either does not sediment completely out of the supernatant solution or sediments through the cushion and is not recovered in the sampling.

Sucrose-Cushion Method. Sample Preparation and Centrifugation. DNA is diluted in solvent and added to the bottom of a centrifuge tube. After delay to allow thermal equilibration, [^{35}S]TF1 (in 0.025 or 0.05 mL) in buffer GD is added to constitute the final sample volume (0.40 or 0.50 mL) and the final solvent composition. After a 15-min incubation, the 0.40-mL sample volume (see assay method) is underlain with 0.10 mL of a 20% sucrose solution in the same solvent, using an Eppendorf pipet with the polypropylene tip placed at the tube bottom. For most experiments, centrifugation is at 40 000 rpm (SW 50.1 rotor, Beckman, 1.92×10^5 g_{max}) for 10 min from the start of acceleration, followed by unbraked deceleration. The total centrifugation, including acceleration and deceleration time, is equivalent (in $\omega^2 t$) to a 12 min centrifugation at maximum velocity.

Sampling. All sampling is with Eppendorf pipets with polypropylene tips. Where the precentrifugation concentration of TF1 is determined, 0.10 mL of the 0.50-mL incubation mixture of [^{35}S]TF1 with DNA is withdrawn after the 15-min incubation, and immediately prior to addition of the sucrose cushion. When the precentrifugation concentration of [^{35}S]-TF1 is not determined, the initial incubation volume is only 0.40 mL.

After centrifugation, the following samples are taken: *sample A* is 50 μL of the lower part of the sucrose cushion, *sample B* is 100 μL of the supernatant volume above the cushion (in duplicate), and *sample C* is 100 μL of a mix of the remaining volume (the mixing is effected by gentle vortex action).

All sampling protocols utilize only one Eppendorf tip for one centrifuge run. In all cases, when the concentration of [^{35}S]-TF1 is varied, those samples with lower TF1 concentration are transferred first. The common postcentrifugation sequence of sampling is the following: the 50-μL cushion samples (A) are removed in order, from the lowest to highest TF1 concentration tubes; the transfer tip is rinsed with a lysozyme solution (1 mg/mL lysozyme in 0.01 M Tris-HCl, pH 7.5) and the duplicate supernatant samples (B) are removed in sequence; the tip is again rinsed in lysozyme solution, the tube contents are mixed, and the mix samples (C) are obtained in sequence. The tip is not rinsed between successive samples in a single sequence.

Determination of Bound [^{35}S]TF1. In the cushion method, TF1 binding can be assayed either by DNA-dependent loss from the supernatant (sample B) or by DNA-dependent entry to the cushion (sample A). Sample C serves as a control of the A sampling. The recovery of TF1 in the cushion sample (A) must be corrected for incomplete recovery of DNA and for [^{35}S]TF1 entry to the cushion sample in the absence of DNA. Native SPO1 and ϕ1 DNAs are completely removed from the supernatant sample (B) during the standard 10-min centrifugation. Under the assay conditions used in the experiments shown in Figures 7 and 8, the recovery of SPO1 DNA from the cushion was 65–85% of the input. In the presence of 0.1% Triton X-100 (Figure 9a), the recovery was somewhat higher. However, at low ionic strength (<0.10), DNA–TF1 complexes sediment through the cushion and are partly recovered in the mixed residue sample (C). When using ^{32}P-labeled SPO1 DNA, the bound [^{35}S]TF1 can be corrected for the exact recovery of DNA in the cushion (Figure 9a). Under the standard assay conditions, denatured DNA does not sediment completely out of the supernatant volume and the centrifugation time was therefore increased to 30 min (Figure 9b).

TF1 binding is measured in experiments in which the concentration of either the TF1 or of the DNA is kept constant and the concentration of the other component is varied. In either experiment, the disappearance of TF1 from the supernatant and its appearance in the pellet are corrected for corresponding changes measured in samples which contain the same concentration of TF1 but have been centrifuged without DNA.

Occasionally, the cushion sample (A) is not precisely located and much of the displaced DNA with its bound TF1 is not recovered in this sample. Measurements on the mix sample (C) allow a correction to be made for this effect. The correction, though less accurate, yields usable data. The correction and its rational are, briefly, as follows. When TF1 is incubated without DNA and is then sedimented, we find a reasonably constant ratio between the counts recovered in the mix sample (C) and the supernatant sample (B). When a large deviation in the C/B ratio is observed after sedimenting TF1 with DNA, we attribute this to the poor recovery of sedimented TF1 from the cushion sample (A). The excess radioactivity in sample C, corrected for the volume of the residual mixture, is added to the cushion-sample (A) estimate of TF1 binding. We have used this correction for several points of Figure 8 (see legend of that figure).

Supernatant Method. The sample is assembled and centrifuged exactly as described above, except that no sucrose cushion is added prior to centrifugation. The TF1–DNA mixture is sampled before centrifugation (100 μL) and two 100-μL samples are taken after centrifugation. This assay method is most useful when the sedimented complex is not recovered in the sucrose cushion sample, and when TF1 loss to the walls of the reaction vessel is not large. We have used this assay in conjunction with a 0.1% Triton X-100 containing solvent, which reduces TF1 loss to the centrifuge tube wall. TF1 bound to DNA is determined by comparing supernatant loss of [^{35}S]TF1 as a result of centrifugation in the presence and absence of DNA. In the absence of DNA, there is a TF1 concentration-dependent loss of TF1 to the centrifuge tube wall. (At 20 °C, comparing pre- and postcentrifugation sam-

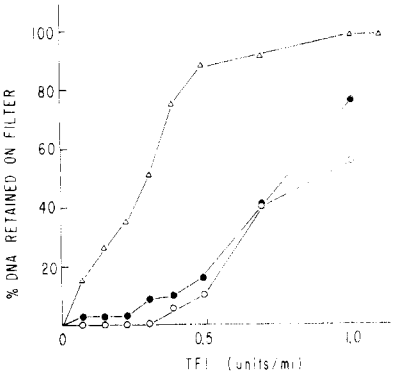

FIGURE 1: Binding of DNA to membrane filters in the presence of TF1. 0.69 μg of DNA in 0.225 mL of buffer TED was brought to 30 °C. 0.025 mL of TF1 in 0.01 M Tris-HCl, pH 7.5, containing 100 μg/mL bovine serum albumin was added and the incubation was continued for 2 min. This incubation mixture was then diluted with 10 mL of ice-cold TED buffer and filtered in a filter holder that had not been cooled. The temperature rose to 11-15 °C during the filtration, which lasted 90-110 s. Serial dilutions of TF1 were made in one test tube by sequentially increasing the volume of diluent. In this way, losses due to adsorption of TF1 to glass were minimized. SPO1 [^{14}C]DNA (△), T4 [^{3}H]DNA (●), and T7 [^{3}H]DNA (○) had specific radioactivities of 1245, 1160, and 1270 cpm/μg, respectively. TF1 was a peak phosphocellulose fraction with a specific activity of 180 units/A_{235}. The concentration of TF1 shown on the abscissa refers to the primary incubation volume of 0.25 mL.

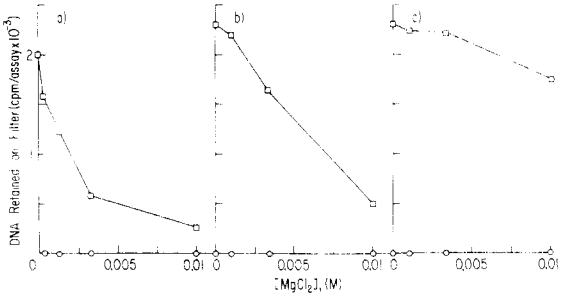

FIGURE 2: Effect of $MgCl_2$ on the TF1-dependent retention of DNA by membrane filters. Each sample contained 0.69 μg of SPO1 [^{14}C]DNA (□) and 0.69 μg of T4 [^{3}H]DNA (○) incubated with 0.3 unit/mL (1.35 μg/mL) of TF1 in the standard binding assay. The incubation solvent was TED with varying concentrations of $MgCl_2$ added. The specific activities of SPO1 and T4 DNA were 3450 and 1160 cpm/μg, respectively. (a) All incubations included 0.01 M $MgCl_2$ and the filtration was with varying concentrations of $MgCl_2$. (b) $MgCl_2$ was present in the incubation medium and diluent at the specified concentration. (c) $MgCl_2$ was present in the preincubation only, at the concentration specified on the abscissa, and no $MgCl_2$ was added for filtration.

ples, the recovery of TF1 averages 97%.)

Centrifuge Tubes. TF1 adsorbs to most reaction-vessel surfaces that we have tested (Johnson and Geiduschek, 1974). This includes common glassware (also siliconized or silanized glass), common centrifuge tubes (nitrocellulose, polypropylene), and several metal surfaces (e.g., stainless steel, plated platinum, or gold). The short-column binding assay uses the least-adsorptive surfaces consistent with centrifugation conditions. These are: (1) a Teflon-S-coated stainless-steel centrifuge tube; and (2) a stainless-steel centrifuge tube, when 0.1% Triton X-100 is included as solvent. We tried thick Teflon inserts in stainless-steel centrifuge tubes, but were discouraged from using them routinely because of their limited lifetime under our conditions of centrifugation.

Handling of [^{35}S]TF1. Dilutions of TF1 were usually made on a Teflon surface (we used a block with carved depressions or a small piece of Teflon sheet). Sequential dilutions of a drop of TF1 on this surface were made by sequential additions of diluent to this one droplet, rather than by serial transfers to several drops of diluent.

For additions of TF1 to DNA, the transfer pipet surface (glass or polypropylene) was equilibrated with the [^{35}S]TF1 solution with at least five sequential filling–emptying cycles. Subsequent transfers of TF1 from this solution yielded a constant quantity of TF1 per aliquot. Wherever constant volumes of increasing TF1 concentrations were to be added, one pipet was used, and the additions were made in sequence, starting with the lowest TF1 concentration. Eppendorf pipets with polypropylene pipet tips were used for all postcentrifugation sampling. As an added precaution, the pipet tip was presoaked in 1 mg/mL lysozyme (in 0.01 M Tris-HCl, pH 7.5), since lysozyme was found to compete with TF1 binding to the pipet tip.

Temperature Control. The following precautions were adopted in order to have relatively constant temperature throughout the incubation and centrifugation time interval. After equilibration of the DNA solution at temperature, TF1 (and the sucrose cushion, if applicable) was added at the incubation temperature. The pipet tip used for the addition was cooled or warmed by successive pipetting of the sample to be added. For experiments at 0–5 and 35 °C, the centrifuge tubes were thermostated in their rotor buckets in a water bath and the rotor buckets were capped, except when material was added or removed. The rotor buckets were affixed to the rotor at room temperature (usually 22–24 °C). Handling was minimized during this period of 2 min (for the addition of the six buckets to the SW 50.1 rotor). The temperature of 0.5-mL samples at 2 °C rises less than 8 °C during this interval and that of samples at 35 °C decreases less than 4 °C. For what we call the 0–5 °C binding experiments, the precentrifugation incubations were at 0 °C and the rotor and rotor chamber were cooled to 0–2 °C. For the 35 °C binding experiment, the rotor chamber was heated to 35 °C with a heat lamp and the rotor was warmed to 46 °C. The sensor of the centrifuge ranged between 2 and 5 °C and 33 and 37 °C during centrifugation, for the low and high temperatures, respectively.

(*9*) *Radioactive Counting.* Radioactivity was routinely determined by spotting labeled fractions on Whatman GF/C glass-fiber filter disks, Whatman 3MM paper disks or nitrocellulose membranes. These disks were dried under a heat lamp and scintillation was counted with "Liquifluor"-toluene.

(*10*) *Other Materials.* The ultrapure grades of Tris base and sucrose from Schwarz-Bioresearch were used for DNA-binding experiments. Dithiothreitol was from Calbiochem and type B-6 nitrocellulose membrane disks were from Schleicher and Schuell. Triton X-100 was from Rohm and Haas; a 1:10 dilution of the Triton X-100 had a conductivity equivalent to 1 mM KCl. All other chemicals were of common reagent grade. Stainless-steel centrifuge tubes were from Beckman (301099, 0.5 × 2.5 in.). Fluorocarbon (Anaheim, Calif.) applied a thin inner surface layer of Teflon-S (Dupont 954-103) to a set of stainless-steel tubes.

Results

Binding of TF1–DNA Complexes to Membranes. TF1 is able to bind SPO1 DNA to nitrocellulose membrane filters. The binding curve of Figure 1 demonstrates a certain degree of specificity, in that TF1 binds native bihelical SPO1 DNA more effectively than it binds T4 or T7 DNA to nitrocellulose filters. The retention of SPO1 DNA–TF1 complexes varies with the $MgCl_2$ content of the incubation and filtration solvents (Figure 2), but the binding of SPO1 DNA remains specific.

TABLE I: Reequilibration in TF1-DNA Binding: Ability of SPO1 DNA to Compete for the TF1 of Preformed TF1-SPO1 DNA Complexes.[a]

μg of Unlabeled SPO1 DNA added with Filtration Solvent	% [^{14}C]SPO1 DNA Retained on Filter[b]
0	100
0.69	102
2.1	90
4.8	66
10.4	32

[a] 0.69 μg of [^{14}C]SPO1 DNA (1500 cpm/μg) was incubated with 0.5 unit/mL TF1 (0.56 μg) in 0.25 mL of TED buffer with 10 μg/mL bovine serum albumin for 2 min at 30 °C. The mixture was diluted with 10 mL of ice-cold TED containing the unlabeled SPO1 DNA, as indicated, and immediately filtered. Filtration time was 70-90 s. [b] Corrected for the retention of [^{14}C]SPO1 DNA in the absence of TF1.

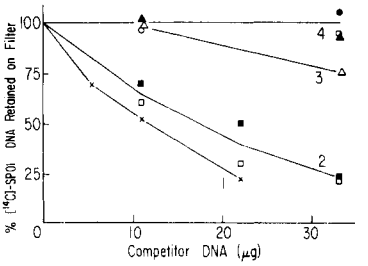

FIGURE 3: Competition assay for TF1-DNA binding. 0.69 μg of SPO1 [^{14}C]DNA (1500 cpm/μg) was incubated with the designated quantity of unlabeled competitor DNA in 0.225 mL of buffer TED. The DNA mixture was brought to 30 °C, and TF1 in 0.025 mL of 0.01 M Tris-HCl, pH 7.5, with 100 μg/mL bovine serum albumin was added to give a final concentration of 0.5 unit/mL (0.56 μg of TF1). The incubation and filtration were as described under Methods, section 6. All of the [^{14}C]DNA is retained on the membrane in the absence of any competitor (100%). All data are corrected for retention in the absence of TF1. DNA was sheared or denatured at 50-100 μg/mL in 0.01 M Tris-HCl, pH 7.5, 0.1 M NaCl. Shearing was done at 0-2 °C by rapidly forcing the DNA solution ten times from a syringe through a 25-gauge hypodermic needle. DNA was denatured in boiling water for 10 min and immediately quenched by immersion in an ice-water bath. DNA symbols: (●) T4; (○) T7; (▲) T4 sheared; (△) T7 sheared; (■) T4 denatured; (□) T7 denatured; (×) SPO1.

At high $MgCl_2$ concentration, the complex is not retained on the membrane filter. Since the presence of $MgCl_2$ in the diluting solvent strongly affects retention, association (in Figure 2c) or dissociation (in Figure 2a) of a retainable complex must occur during the time span of the filtration, either in solution or on the filter itself.

While determining the requirements for membrane filter retention of the TF1-DNA complex, we discovered kinetic properties of these TF1-DNA complexes which must complicate the quantitative interpretation of nitrocellulose filter assays in terms of DNA-protein equilibria. The following observations have been made.

(1) Preincubation of TF1 with DNA at 30 °C, according to the standard assay (Methods section 6), leads to maximal DNA retention; however, an almost equivalent quantity of SPO1 DNA is retained if one adds the same amount of TF1 at a 40-fold lower concentration, after dilution but immediately before filtration (data not shown). When TF1 is only present in the primary 0.25-mL incubation at this lower concentration (Figure 1), there is almost no retention of SPO1 DNA on the filter. Prefiltering of TF1 through the nitrocellulose filter does not enhance binding of subsequently filtered SPO1 DNA (data not shown).

(2) The DNA of a TF1-DNA complex that is trapped on the membrane filter is rapidly lost with additional rinsing. For example, when complexes were formed and filtered as under Methods, section 6, but then washed with additional TED buffer at 5 mL/min, DNA was continuously removed at a relatively rapid rate (85% retention in the standard assay; 50% of this retained DNA removed by additional rinsing for 1.5 min).

(3) If the filtration rate of a TF1-DNA complex is merely slowed, the retention of DNA by the membrane is decreased (data not shown) albeit very much more slowly than in the preceding experiment. A simple calculation (involving assumptions of first-order elution of complex from the filter and instantaneous equilibration in solution) confirms that rinsing the filter with buffer causes more rapid elution of already bound DNA than does rinsing with buffer containing more SPO1 DNA and TF1. Effects 1, 2 and, to a lesser extent, 3 must occur during our standard assay, which involves a dilution and a 90-s filtration.

Two possible sources of the above effects must be considered: (1) rapid dissociation and reequilibration of TF1-DNA complexes and (2) inactivation of the DNA-binding activity of TF1 on nitrocellulose filters. The experiment of Table I suggests that reequilibration of TF1-DNA complexes must be rapid, and further evidence is provided below. Our experiments do not provide any information about TF1 inactivation on the filter.

In order to study the interaction of TF1 with various DNAs, the filter binding experiment was modified into a competition assay, in the following way. An unlabeled competitor DNA was mixed with SPO1 [^{14}C]DNA prior to the addition of TF1. A quantity of TF1 was then added which could completely bind the SPO1 [^{14}C]DNA by itself, and the whole sample was treated as already described. Since binding of the SPO1 [^{14}C]DNA is dependent on the TF1 concentration (Figure 1), this is a direct test of the ability of the unlabeled competitor DNA to bind TF1 and thus to lower the free TF1 concentration. The binding-competition curve of unlabeled and labeled SPO1 DNA is shown in Figure 3, curve 1, and serves as a standard of comparison for competition experiments involving labeled SPO1 DNA and unlabeled heterologous DNA. Various competition curves of this kind are shown in Figure 3. Even at the highest concentrations used, T4 and T7 DNAs (curve 4) do not show marked competing ability and, evidently, do not decrease the concentration of TF1 available for retention of SPO1 [^{14}C]DNA on the membrane; in contrast, 2.5-fold less unlabeled SPO1 DNA competes 50% of labeled-DNA binding. Thus, in this solvent, TF1 binds to T4 and T7 DNA with lower affinity, or at fewer sites, or both. Denatured T4 and T7 DNA (curve 2) both compete relatively effectively for binding of TF1 to helical SPO1 [^{14}C]DNA. Either the denatured T4 and T7 DNA binds TF1, or else it competes at the nitrocellulose membrane for TF1-dependent DNA binding. The latter explanation is distinctly less plausible, because these membranes are known to have capacities for denatured-DNA binding which far exceed the quantities used in this experiment. Shearing double-stranded T4 and T7 DNAs slightly increases its competing activity (curve 3), perhaps because of some shear denaturation and uneven breakage of double strands (Hershey et al., 1963). Denatured SPO1 DNA competes approximately as effectively as helical SPO1 DNA (data not shown). However, we found a considerable variability of the competing

power of this DNA, for reasons that we do not understand. Within this limitation, denatured DNAs do not differ substantially in their binding of TF1, as measured by the competition assay.

Further evidence for rapid equilibration of TF1 between SPO1 DNA molecules comes from a competition experiment in which we examined whether the order of interaction of labeled and unlabeled SPO1 DNA with TF1 affects retention of labeled DNA by filters (Table I). Evidently, unlabeled DNA added with the diluent competes effectively for TF1 previously bound to labeled DNA. The competition is rapid, since the competitor DNA is added immediately before filtration and since filtration only lasts 70–90 s.

Sedimentation Properties of TF1–DNA Complexes. In the next experiments, we have examined TF1–DNA complexes by sucrose density gradient sedimentation. TF1 and DNA were mixed and the DNA was centrifuged away from unbound TF1 through a sucrose gradient substrate so that TF1 and DNA displacements could be measured. While the filter-binding experiments had been in buffer TED, these experiments were done in the presence of added KCl, in buffer TKE (at ionic strength 0.18) and isothermally, at 20 °C. The result of one series of experiments is shown in Figure 4. Here a constant amount of TF1 was added to increasing quantities of SPO1 DNA and the [^{3}H]TF1 displacement with unlabeled SPO1 DNA (Figure 4) and the SPO1 [^{14}C]DNA displacement with unlabeled TF1 (data not shown) were measured in separate experiments. The major difficulty in this experiment is the tendency of TF1 to bind to a variety of surfaces. As noted in the caption of Figure 4a, the recovery of TF1 from the gradient is low and depends on DNA concentration: the surfaces of vessels, in which the TF1–DNA complex is assembled, transferred, and centrifuged, are in competition with DNA for binding free TF1. Evidently, TF1–DNA complexes do not bind tightly to these surfaces since the recovery of [^{14}C]DNA is high. The amounts of TF1 sedimenting with DNA and the total TF1 displacement from the meniscus have been estimated and are compared in Figure 4b. At high DNA concentration, almost all the recovered TF1 is displaced with, and therefore bound to, SPO1 DNA. Evidently, total displacement from the meniscus (curve 1) considerably exceeds the amount remaining with the DNA (curve 2), especially at lower DNA concentrations. Thus, even during 15-min sedimentation, a large fraction of TF1 initially bound to the DNA has dissociated from the rapidly sedimenting TF1–DNA complex as it sediments through the sucrose. In view of this rapid loss of bound TF1 from sedimenting DNA, we tested, in the next experiment, for the ability of DNA-bound and unbound TF1 to exchange. [^{3}H]TF1 and SPO1 or ϕ1 DNAs were sedimented in three different ways (Figure 5). In experiment a, 0.3 μg of [^{3}H]TF1 and 1.1 μg of DNA were applied to gradients and sedimented. A distinct peak of DNA-bound TF1 is found only with SPO1 DNA and not with ϕ1 DNA; this shows the expected specificity of binding of TF1. An additional quantity of TF1 is displaced from the applied zone (cf. Figure 5a with 5b or 5c) by both SPO1 and ϕ1 DNAs. For SPO1 DNA, this repeats the result of Figure 4. It is striking that ϕ1 DNA displaces a substantial amount of loosely bound TF1 from the meniscus. While this might be taken to imply the existence of two kinds of binding sites for TF1 on SPO1 DNA—specific and nonspecific—one can also offer alternative interpretations. We shall see in the next section that quantitative measurements of TF1–DNA binding, which are the best way of pursuing this question, do not provide evidence for two types of binding sites on SPO1 DNA for TF1. In Figure 5b, 1.5 μg of [^{3}H]TF1 has

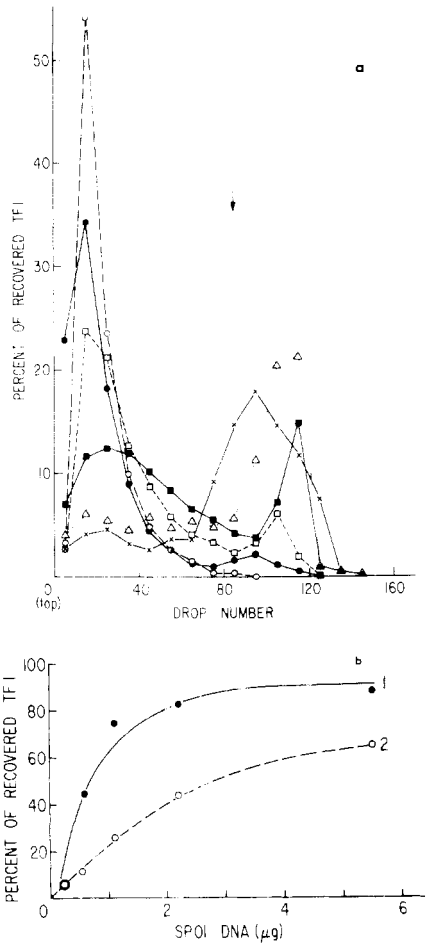

FIGURE 4: Sedimentation of TF1 with SPO1 DNA. 0.2-mL samples containing 1.2 μg of [^{3}H]TF1 and the designated quantities of DNA in buffer TKE were placed on 5.0-mL 5–20% sucrose gradients in the same buffer. Centrifugation was in nitrocellulose tubes at 20 °C. The rotor (Beckman, SW 50.1) was accelerated to 45 000 rpm, centrifuged for 15 min from the beginning of acceleration, and allowed to come to rest without braking. Samples were collected from the tube bottom. Sedimentation is from left to right in the figures, and only the top half of the gradients is shown (total of 300–320 drops per gradient; no TF1 or DNA was recovered in the lower fractions). Native SPO1 DNA, sedimented without added TF1, would be centered (↓) at drop 85. (a) Distribution of TF1 in the gradient. Recovery of [^{3}H]TF1 in the gradient fractions was 17, 30, 41, 50, 56, and 55% for no DNA, 0.22, 0.55, 1.1, 2.2, and 5.5 μg of DNA, respectively. At the lower DNA concentrations, up to 20% of the added [^{3}H]TF1 was recovered in the glass tube used for combining the zone sample components. In a parallel experiment with SPO1 [^{14}C]DNA and unlabeled TF1, for which the data are not shown, the recovery of DNA from the gradient ranged from 75 to 82%. (○) No DNA, (●) 0.22 μg of DNA; (□) 0.55 μg of DNA; (■) 1.1 μg of DNA; (△) 2.2 μg of DNA; (×) 5.5 μg of DNA. (b) TF1 displacement and residual binding to SPO1 DNA. Curve 1 (●): total TF1 displacement; curve 2 (○): TF1 remaining bound to DNA after centrifugation. The data of (a) have been analyzed as follows. In each TF1 distribution, the TF1 remaining in the two fractions nearest the meniscus (drops 1–20) is assumed to have been unbound. From the distribution profile of TF1 in the absence of DNA (Figure 4a, open circle) and the ratio (fraction of TF1 in drops 1–20 in presence of DNA)/(fraction of TF1 in drops 1–20 in absence of DNA), a profile of unbound TF1 in the originally applied supernatant zone, in the *presence* of DNA has been calculated for each DNA concentration. By subtracting this calculated distribution for unbound TF1 from the observed distribution, we have derived the distribution of total displaced TF1 for each sample, added up all the displaced radioactivity, and plotted the sum for each sample in curve 1 (●). We have also assumed that the TF1 under the DNA peak, i.e., within the zone of sedimenting DNA, is partly composed of dissociating TF1. The latter has been estimated by extrapolating the distribution of total displaced TF1 (measured outside the DNA zone) across the DNA zone. The difference (total TF1 in the DNA zone) − (dissociating TF1 in the DNA zone) is the TF1 that remains bound to the DNA after centrifugation and is plotted in curve 2 (○).

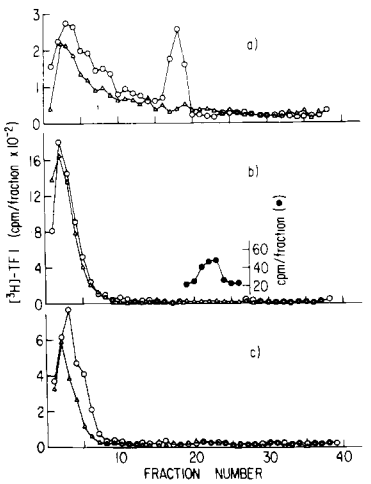

FIGURE 5: Sedimentation of [^{3}H]TF1 with SPO1 and ϕ1 DNAs. The solvent, sucrose gradient, sample volume, and fractionation method were as described in the legend to Figure 4. Centrifugation was in the SW 50.1 rotor at 17 000 rpm and 20 °C for 240 min. (a) 0.29 μg of [^{3}H]TF1 (4800 cpm/μg of TF1) was centrifuged with 1.1 μg of SPO1 DNA (○) or 1.1 μg of ϕ1 DNA (△) in two separate gradients. (b) 1.5 μg of [^{3}H]TF1 (4800 cpm/μg of TF1) was mixed with 15 μg of unlabeled TF1 (final 436 cpm/μg of TF1) and centrifuged with 1.1 μg of SPO1 DNA (○, ●) or 1.1 μg of ϕ1 DNA (△) in two separate gradients. The right-hand expanded scale shows a small peak of TF1 radio activity (●) sedimenting with SPO1 DNA. (c) 0.29 μg of [^{3}H]TF1 (4800 cpm/μg of TF1) was centrifuged with 1.1 μg of SPO1 DNA (○) or 1.1 μg of ϕ1 DNA (△) through gradients containing 10.8 μg/mL unlabeled TF1.

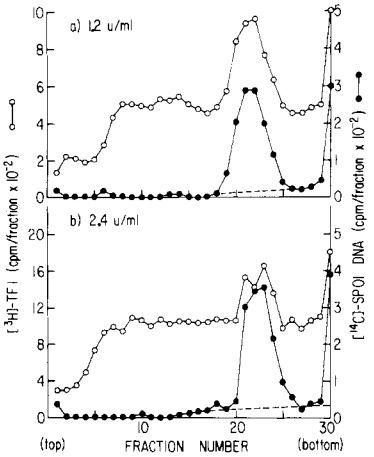

FIGURE 6: Sedimentation of SPO1 DNA through TF1 gradients. Ten micrograms of SPO1 [^{14}C]DNA (188 cpm/μg) and 6.4 μg of [^{3}H]TF1 (485 cpm/μg) in a 0.2-mL zone sample in buffer TKE were sedimented through 5–20% sucrose gradients in the same buffer, containing 1.2 or 2.4 units/mL [^{3}H]TF1 (5.5 or 10.9 μg/mL) of identical specific radioactivity (485 cpm/μg). Centrifugation was at 15 200 rpm for 240 min at 20 °C in nitrocellulose tubes in the Beckman SW 50.1 rotor. Ten micrograms of SPO1 DNA sedimented to fraction 8 in the absence of TF1. Bound TF1 was calculated (see text) for fractions 20–24 in the top panel and 21–24 in the bottom panel. For each gradient, the bottom fraction (fraction 30; the "pellet") was also analyzed for SPO1 DNA and excess [^{3}H]TF1.

been isotopically diluted tenfold with unlabeled TF1 before mixing with DNA. Since the total quantity of TF1 mixed with the DNA is in vast excess of the binding capacity of the latter, very little radioactivity sediments down the gradient. What little does sediment, moves further in experiment b than in experiment a, because the TF1/DNA ratio is greater and because the sedimentation rate of DNA increases as the TF1/DNA ratio increases (see below). For the samples shown in panel c, the excess TF1 has been dispersed in the sucrose substrate and the specific activity and quantity of [^{3}H]TF1 applied in the zones together with the DNA are the same as in panel a. No radioactivity sediments down the gradient. Moreover, the distribution of [^{3}H]TF1 radioactivity near the meniscus in panel c resembles that in panel b, and there is much less spreading of radioactivity down the gradient than in panel a. Clearly, the DNA-bound radioactive TF1 exchanges readily with unlabeled TF1, leaving the radioactivity at the top of the gradient. Exchange (panel c) removes labeled TF1 from the zone of sedimenting DNA more effectively than does dissociation (panel a). Figure 5 and Table I show rapid reequilibration of DNA and TF1 ligands in DNA–TF1 complexes.

In the next series of experiments, we examined the influence of TF1 on the sedimentation rate of DNA. TF1 was mixed with DNA and then centrifuged through gradients that either contained, or were free of, TF1 for varying periods of time. In a control experiment, the sedimentation of free SPO1 DNA was separately measured. The SPO1 DNA–TF1 complexes, which were sedimented into buffer lacking TF1, started out at a rapid rate but slowed down as they shed TF1 (compare Figure 4a) and finally approached the sedimentation rate of free DNA. In contrast, when sedimenting through a substrate containing TF1 at the concentration of the supernatant zone, the sedimentation rate of the SPO1 DNA remained constant and high. We infer that the rapid sedimentation of the SPO1 DNA–TF1 complex occurs only with relatively high TF1 concentrations; it is not sufficient to have relatively high affinity binding of a few TF1 molecules to a limited number of DNA sites. In other experiments, we measured the sedimentation rate of SPO1 DNA as a function of TF1 concentration in buffer TKED at 20 °C, using TF1-containing sucrose gradients. At the highest TF1 concentrations used (2.4 units/mL), the average sedimentation rate of SPO1 DNA was increased threefold.

With this information on the sedimentation of DNA and TF1 at hand, the following experimental arrangement was used to estimate the capacity of SPO1 DNA for binding TF1: [^{3}H]TF1 and SPO1 [^{14}C]DNA were mixed in the zone sample and their complex was sedimented through [^{3}H]TF1-containing sucrose gradients. The excess TF1 in the zone of sedimenting DNA was taken to be bound TF1 (Figure 6).

The DNA concentration was relatively high (50 μg/mL in the applied zone) in order to have the concentrations of bound and free TF1 in the sedimenting zone comparable. A fraction of the DNA (up to 20% of the total at the highest TF1 concentration) pelleted with bound TF1 and afforded another measurement of TF1 binding. The depletion of TF1 at the meniscus is due to removal with DNA, rather than free sedimentation of free TF1. TF1 concentrations in the gradient, in the range of 0.4 to 2.4 units/mL (1.8–10.8 μg/mL), gave TF1–DNA complexes that sedimented 2.8–3.2 times as rapidly as pure DNA. At the higher TF1 concentration (1.2 and 2.4 units/mL), an average of 0.60 μg of TF1 (0.64 and 0.57 μg, respectively) was bound per μg of DNA in the sedimenting zone, while the pelleting DNA bound 0.72 μg of TF1 (0.70 and 0.74 μg respectively) per μg of DNA. We regard these binding coefficients as being no more accurate than $\pm$0.15 μg/μg. The principal uncertainties come from the measurement of free TF1, for which the chosen value is indicated as a dotted line in Figure 6 and the DNA, for which a baseline is also indicated as a dashed line.

Measuring TF1–DNA Binding by Short-Column Centrifugation. For the first experiment, we examine binding of TF1 to ϕ1 and SPO1 DNAs at 20 °C. SPO1 or ϕ1 DNA was equilibrated in 0.375 mL of buffer in a Teflon-S-coated

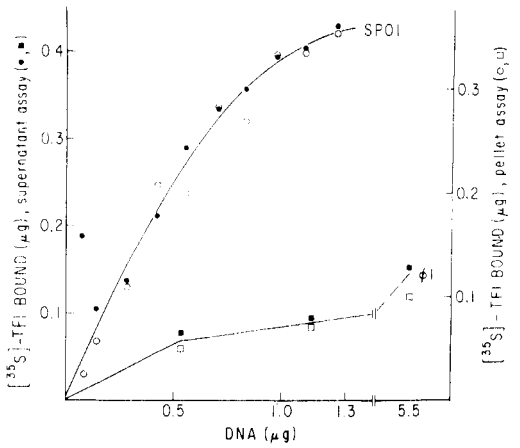

FIGURE 7: Short-column centrifugation of TF1–DNA complexes. Selective binding of ^{35}S-labeled TF1. Each sample was composed as follows: 0.375 mL of buffer containing varying quantities of DNA (abscissa) were equilibrated in six Teflon-S-coated stainless-steel 5-mL centrifuge tubes at 20 °C. 0.025 mL of buffer GD containing 1.04 μg of TF1 (45 000 cpm/μg) was then added. The 0.4-mL of mixture then had the composition of buffer TKED (0.1 M Tris-Cl, pH 7.5, 0.1 M KCl, 0.1 mM EDTA, 0.06 mM dithiothreitol, ionic strength 0.18). Each sample was then underlain with 100 μL of 20% sucrose (w/v) in buffer TKE, the centrifuge rotor (Beckman SW 50.1) was loaded, and centrifugation for 10 min at 40 000 rpm was carried out as described under Methods, section 8a. After centrifugation, the following samples were obtained in sequence: 50 μL from the sucrose cushion, taken from the bottom of the tube, 100 μL from the supernatant, and 100 μL from a gentle vortex mixture of the remaining material. [^{35}S]TF1 binding to SPO1 DNA (○, ●) or φ1 DNA (□, ■) was calculated by sucrose-cushion entry (open symbols) or supernatant loss (closed symbols). Recovery of added [^{35}S]TF1 averaged 70% (range 59–75%).

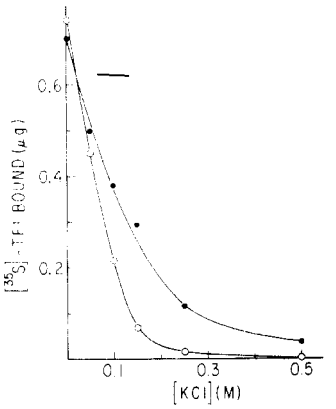

FIGURE 8: Effect of ionic strength on [^{35}S]TF1 binding to DNA. 0.55 μg of SPO1 or φ1 DNA in 0.375 mL of phosphate buffer with the desired concentration of KCl was brought to 20 °C in a centrifuge tube. 0.025 mL of [^{35}S]TF1 (1.4 μg, 42 500 cpm/μg) in buffer GD was added, yielding 0.40 mL of sample with 10 mM phosphate (Na), pH 7.5, buffer and the KCl concentrations indicated on the abscissa. The ionic strength contributed by the phosphate buffer is 0.025. Fifteen minutes after the addition of TF1, each sample was underlain with 100 μL of 20% sucrose (w/v) in 10 mM phosphate (Na), pH 7.5, with the indicated KCl concentration. The Teflon-S-coated stainless-steel tubes were centrifuged and sampled to determine the [^{35}S]TF1 distribution (sucrose cushion assay; Methods, section 8). In the absence of DNA, [^{35}S]TF1 entry to the sucrose-cushion sample averaged 0.026 μg of [^{35}S]TF1, independently of ionic strength. The ratio of the mix sample (C) to the supernatant sample (B) (see Methods, section 8) averaged 0.81 (range 0.76–0.86) in the absence of DNA. A major correction to the point for SPO1 DNA, 0.10 M KCl, where this observed ratio is 2.2, has been made. [^{35}S]TF1 bound to SPO1 (●) or φ1 (○) DNA is shown on the ordinate.

stainless-steel centrifuge tube at 20 °C, and 0.025 mL of [^{35}S]TF1 (1.04 μg, 45 000 cpm/μg) was added to yield the final TKED solvent (ionic strength, 0.18). After centrifugation, samples were obtained in sequence from the sucrose cushion, from the supernatant, and after a gentle vortex mix of the remaining liquid volume.

TF1 binding to DNA has been calculated on the following bases. (1) Entry of [^{35}S]TF1 to the cushion sample in the presence of DNA, corrected for the entry of [^{35}S]TF1 to the cushion sample in the absence of DNA (Figure 7, symbols ○, □), and (2) recovery of [^{35}S]TF1 in the supernatant sample in the presence of DNA corrected for recovery of [^{35}S]TF1 in the absence of DNA (symbols ●, ■). Binding of [^{35}S]TF1 is proportional to SPO1 DNA concentration up to 0.6 μg (1.5 μg/mL), with the sucrose-cushion entry estimate of binding approximately 80% of the supernatant estimate. We show below that this lower estimate is due to incomplete recovery of the DNA in the sucrose-cushion sample under these experimental conditions (Figure 9). The near equivalence of the sucrose cushion and supernatant sample estimates of bound TF1 implies that the sampling method does not drastically disrupt the density layers in the centrifuge tube. The experiment shown in Figure 7 clearly confirms that, under the conditions of this experiment, TF1 binds preferentially to SPO1 DNA relative to φ1 DNA.

(Note: The method assumes that the concentration of TF1 remaining in the supernatant is equal to the concentration of free TF1 in equilibrium with TF1–DNA complexes before centrifugation and that the addition of SPO1 DNA causes only DNA-bound TF1 to sediment into the pellet. This assumption is not precisely valid if reequilibration of ligands occurs during centrifugation (Schachman, 1959; Cann, 1970). Such reequilibration would ensue if a partial separation of zones of DNA and DNA–TF1 were to occur. For example, if DNA were to lag behind DNA–TF1 complexes in the sedimentation, then the quantity of TF1 swept out of the supernatant during sedimentation would exceed the quantity bound at equilibrium and at rest. As a consequence, the affinity of DNA for TF1 would be overestimated. However, we found that, when preformed complexes of DNA and TF1 were sedimented through *subsaturating* concentrations of TF1 (in experiments like those shown in Figure 6 for saturating TF1), the zones of DNA and of DNA-bound TF1 were superimposed and sedimented together, and there was no consistently observed asymmetry of distribution of TF1 within the sedimenting DNA zone. Therefore, we conclude that there is no substantial redistribution of TF1 during the short-column centrifugation and that our method of determining TF1 binding is valid.)

For the next experiment, which tests the ionic-strength dependence of TF1 binding to DNA, we used 0.01 M phosphate (Na), pH 7.5, buffer and increased the ionic strength with KCl (Figure 8). TF1 binding was measured by the sucrose-cushion assay method (Methods, Section 8 and Figure 8 legend): 1.4 μg of [^{35}S]TF1 (in 0.025 mL) was added to 0.55 μg of SPO1 or φ1 DNA (in 0.375 mL of the appropriate solvent) to yield a final solvent with 0.01 M phosphate (Na), pH 7.5 buffer and the designated KCl concentration. It is apparent from this experiment that substantial *and* selective binding of TF1 to SPO1 DNA is observed over a relatively limited ionic strength range (0.1–0.2). At lower ionic strength (≤0.05 M KCl), apparently equivalent binding to φ1 and SPO1 DNA occurs. However, although the single TF1 and DNA concentrations used here do not show it, the binding of TF1 to SPO1 and other DNAs may not be equally tight, since membrane filter assays do distinguish the binding of different DNAs at lower ionic strength (Results, section 1). In this experiment, in the absence of KCl, the quantity of bound TF1 is 5.4 times the quantity of

free TF1: 1.3 μg of [^{35}S]TF1 binds per μg of SPO1 or φ1 DNA in the presence of only 0.33 μg/mL free TF1. Presumably, the capacity of DNA for TF1 at this ionic strength is even greater than the above value. The variation of TF1 binding with ionic strength also underestimates the dependence of affinity on ionic strength, because this experiment is done at constant total, rather than free, TF1 concentration. Indeed, the ionic strength of the incubation, in addition to affecting the affinity of DNA for TF1, also influences the recovery of TF1. In the absence of DNA, the low ionic strength (no KCl) recovery of TF1 is only 77% of that recovered at high ionic strength (0.25 M KCl). All of these factors combine to make Figure 8 present the reader with an *under*estimate of the dependence of TF1 binding on ionic strength. Seeing nonselective binding of TF1 at low ionic strength, we thought that one might be able to expose a selective component of TF1 binding in the short-column centrifugation assay, even under these low ionic strength conditions, by adding a great excess of a basic protein that should bind electrostatically and nonselectively. Lysozyme (isoelectric point 11–11.2) was chosen for this attempt. In the presence of a 36-fold excess of lysozyme (50 μg relative to 1.4 μg of TF1), the binding of TF1 at the lowest ionic strength to SPO1 and φ1 DNAs was reduced by almost a factor of two, but the binding remained nonselective. At higher ionic strength (>0.1 M KCl), lysozyme did not substantially reduce the binding of TF1 to either DNA.

In the next experiment, the binding of TF1 to SPO1 DNA has been measured at 20 °C in buffer PK in the presence of 0.1% Triton X-100. The function of Triton is to reduce surface binding of TF1. It does not block RNA synthesis or the transcription-inhibitory activity of TF1. In one part of this experiment (Figure 9), different constant quantities of TF1 were mixed with varying quantities of DNA before analysis by short-column centrifugation (supernatant method). At each total concentration of TF1, a sample without DNA served as the control for loss of TF1 from the supernatant without DNA binding. The initial steps of these binding curves provide estimates of TF1 binding to SPO1 DNA at the appropriate concentrations of TF1. For the experiment shown in Figure 9a, SPO1 [^{32}P]DNA was used, and TF1 binding was determined (sucrose cushion assay) both in terms of loss of TF1 from the supernatant and in terms of accumulation in the cushion phase. For the latter, TF1 binding was corrected for ^{32}P recovery in the cushion sample. The two assays of binding, corrected in this way, are seen to agree, confirming the validity of the scaling used in Figure 7.

The binding of TF1 to varying quantities of native and denatured SPO1 [^{32}P]DNA is compared in Figure 9b. For the denatured SPO1 DNA, the centrifugation time was increased to 30 min to allow more complete removal of the denatured DNA from the supernatant volume. Under these conditions, the binding of TF1 to native and denatured DNAs does not differ greatly. Binding experiments were also done with constant amounts of DNA and TF1 concentration varying. The short-column centrifugation runs were analyzed by the supernatant method, and the control for loss of TF1 consisted of corresponding samples lacking DNA. The results of this experiment are shown in Figure 10, together with appropriate entries from the data of Figures 7 and 9 (intercepts of the binding curves for 0.55 μg of DNA; closed squares and closed circle). We estimate the capacity of SPO1 DNA for TF1 in buffer PK to be 0.57 μg of TF1/ μg of SPO1 DNA. One molecule of the 23 000 molecular weight TF1 (Johnson and Geiduschek, 1972) binds, on the average, to every 60 nucleotide pairs of SPO1 DNA. At the highest TF1 concentrations, the

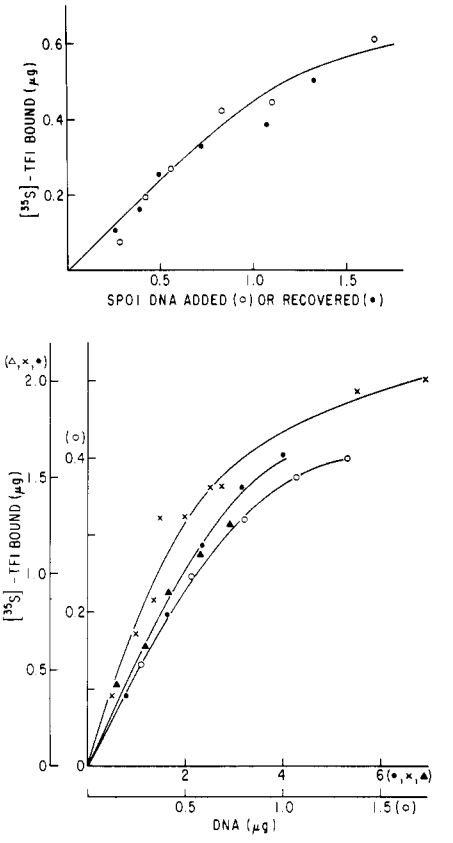

FIGURE 9: Binding of TF1 to SPO1 DNA at 20 °C in PK buffer with 0.1% Triton X-100: Constant TF1; DNA concentration varying. Varying quantities of SPO1 DNA and various constant amounts of TF1 were preincubated, centrifuged, sampled, and assayed as described under Methods, section 8, and in the captions of preceding figures. (a, top) 0.99 μg of TF1 (2.49 μg/mL) and varying quantities of ^{32}P-labeled SPO1 DNA were incubated in 0.4 mL of buffer PK at 20 °C, centrifuged, and sampled from the cushion and supernatant. No residual ^{32}P was detected in the supernatant (e.g., <2% of the label with 1.07 μg of SPO1 DNA) after centrifugation. The recovery of ^{32}P in the cushion sample was on the average 87% of that anticipated for complete sedimentation into that phase. Coordinates on the abscissa have been corrected for incomplete recovery of DNA in individual cushion samples (●). Supernatant loss assays (○). (b, bottom) DNA concentration—varying TF1 binding curves for the following total concentrations of TF1—1.66 (○), 7.7 (×), and 7.9 (●) μg/mL with helical DNA. Sampling of supernatant loss only. Interpolated values of TF1 binding for 0.55 μg of DNA are entered in Figure 10. Binding of TF1 (8.4 μg/mL; ▲) to denatured SP01 [^{32}P]DNA. Samples containing denatured DNA were centrifuged for 30 instead of 10 min. Sampling of supernatant loss only. Binding of TF1 to denatured DNA was corrected for incomplete removal of DNA from the supernatant.

blank corrections are sufficiently inaccurate and the data scatter sufficiently to make the estimate of the capacity relatively inaccurate. Half-maximum binding occurs at a free TF1 concentration of 0.6 μg/mL, implying an affinity constant, K_a, of 1.7 mL/μg in a Langmuir isotherm approximation. Taking the binding species of TF1 to be the 23 000 molecular weight dimer, $K_a = 3.9 \times 10^7$ L/mol. However, the binding isotherm is not adequately described in these terms (note the fit of curve 1 in Figure 10 to the binding data). Further analysis is presented below.

The temperature dependence of TF1 binding to SPO1 DNA in PK–Triton has also been measured (at 0–5, 20, and 35 °C). The short-column centrifugations were analyzed by the supernatant method with equivalent DNA-less samples of TF1 run as controls at each temperature. The temperature dependence of binding is not great and binding is greater at 20 °C than at 5 or 35 °C (Figure 11). The lower binding at 35 °C

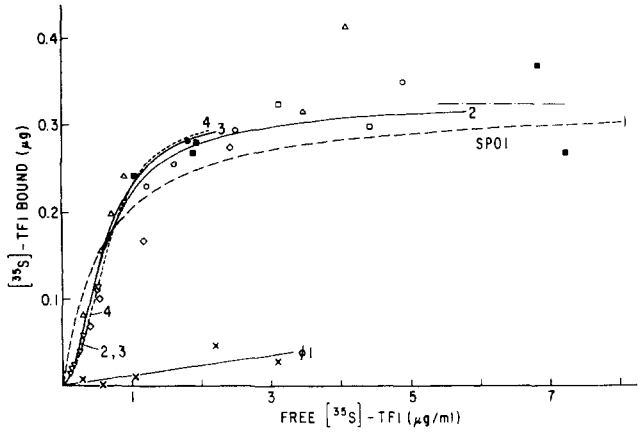

FIGURE 10: Binding isotherm of TF1 to SPO1 and ϕ1 DNAs at 20 °C in buffer PK with 0.1% Triton X-100. Each sample contained 0.55 μg of SPO1 or ϕ1 DNA or no DNA and varying quantities of TF1 in 0.4 mL of buffer PK containing 0.1% Triton X-100. Sample preparation, centrifugation, and sampling (supernatant only) were as described under Methods (section 8b). Loss of TF1 from the supernatant phase in the presence of DNA was corrected through the analysis of correspondingly treated samples containing no DNA. Symbols: different open symbols refer to different centrifugation runs with SPO1 DNA. Closed symbols refer to binding data interpolated from constant TF1–DNA varying binding data for this quantity (0.55 μg) of SPO1 DNA. Crosses refer to ϕ1 DNA binding. (◇) Data from 20 °C experiment, Figure 11 (see below). (■) Data from Figure 9 (and one similar experiment not shown in Figure 9). (●) Data from Figure 7; solvent TKED (ionic strength 0.18, almost same as PK which is 0.175). Calculated curves: (1) Langmuir isotherm, K_a = 1.7 mL/μg; (2) dissociating ligand, K_2 = 0.1 mL/μg, K_a = 9.1 mL/μg; (3) cooperative binding, K_a = 0.4 mL/μg, ω = 4; (4) cooperative binding, K_a = 0.3 mL/μg, ω = 5. The capacity for all the calculated curves is taken to be 0.58 μg of TF1/μg of DNA. (The capacity is indicated as a dashed horizontal line.)

might be related to TF1 dissociation or to the previously analyzed reversible changes of TF1 secondary structure, which already occur at this temperature (Johnson and Geiduschek, 1972). These very slight temperature effects on binding, though not analyzed in detail, argue against a requirement of SPO1 DNA unwinding for selective binding of TF1. Similar experiments were also done in PK solvent without Triton. In this case, losses of TF1 during the experiment were particularly severe at 35 °C and were corrected for in a relatively laborious way. The results (not shown) were comparable: there was more binding at 20 than at 0–5 °C; binding at 35 °C was the lowest. At 20 °C, TF1 binding in the presence and absence of Triton was comparable. for some runs at 20 °C, we also used a sample of SPO1 DNA that had been purified by equilibrium centrifugation in CsCl. The binding of TF1 to this DNA and to DNA purified in the normal way by phenol extraction did not differ substantially (Figure 11).

Discussion

TF1 is a DNA-binding protein with a degree of specificity for SPO1 DNA, a DNA which contains the modified pyrimidine, hydroxymethyluracil, in place of thymine. But TF1 is a basic protein and it is therefore not unexpected that it also has some affinity for any DNA. In this paper, we have studied some aspects of this binding. The nature of the TF1–DNA interaction and the peculiarities of TF1 pose two kinds of practical problems. One set of problems is posed by the surface adhesiveness of TF1. Some of the experiments in this paper are, to some degree, marred by this problem. We have not yet found any entirely nonadsorptive surface for dilute aqueous TF1 solutions. However, Teflon is better than any other that we

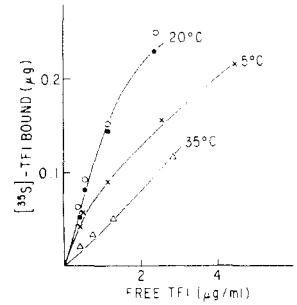

FIGURE 11: Effect of temperature on binding of TF1 to SPO1 DNA. Each sample contained 0.5 μg of SPO1 DNA or no DNA and varying quantities of TF1 in 0.4 mL of buffer PK with 0.1% Triton X-100. Sample preparation, centrifugation, and sampling (supernatant only) were as described under Methods, section 8b. Handling of samples at 0–5 and 35 °C was as described under Methods, section 8e. 20 °C (○, ●); 5 °C (×); 35 °C (△). One set of samples (●) was prepared with CsCl-purified SPO1 DNA.

have tried and stainless steel, as well as KelF, are superior to glass, while nitrocellulose is the least suitable (Johnson and Geiduschek, 1974). Apart from that, we have tried to handle TF1 as far as possible in concentrated solution and with the minimum number of transfers. The surface adhesiveness of TF1 is substantially decreased when the neutral detergent Triton X-100 is incorporated into the solvent buffer, and several of the experiments reported in the preceding section have been done in this way. Another set of problems is posed by the relatively weak binding of TF1 to SPO1 DNA. This is associated with rapid reequilibration of DNA–TF1 with free DNA or free TF1, and with a rapid dissociation of DNA–TF1 when free TF1 is removed. The time scale of that dissociation is so short that it renders the classical trapping method, nitrocellulose filter binding, unsuitable for quantitative work. For example, the experiment summarized in Table I shows that TF1–DNA complexes can reequilibrate with DNA in a time that is short compared to the filtration time of 90 s. A rough calculation based on Table I suggests that the first-order dissociation rate constant must be at least 0.12 s^{-1} (and it may be very much greater; see the note inserted as the next paragraph). Nevertheless, the filter-binding assays did permit us to demonstrate that a degree of specificity exists in the binding, even at lower ionic strength in buffer TED (Figures 1 and 3).

(Note: The calculation is the following. A complex between labeled DNA and TF1 has been preformed under such conditions that n molecules of TF1 bind to each molecule of SPO1 DNA; at t = 0, a trap for free and dissociating TF1 is added and filtration through a membrane filter is started simultaneously. The filtration is terminated t seconds later. A molecule of labeled SPO1 DNA is assumed to remain bound to the membrane filter if at least one TF1 ligand is still attached at the end of the filtration. The decay time of a complex at a single DNA site is χ. Decay of complex is assumed to occur at the same rate on the nitrocellulose filter and in solution (there is no assurance that this assumption is correct; the RNA polymerase–DNA complex dissociates more rapidly on membrane filters than in solution).

The probability that any single originally occupied TF1 binding site on DNA is unoccupied at time t is

$$1 - e^{-t/\chi}$$

The probability that all n sites are unoccupied is

$$(1 - e^{-t/\chi})^n$$

so that the probability that at least one of n binding sites is still occupied at time t, which is also the fraction of labeled DNA, f, still bound to the filter in the presence of excess unlabeled competitor DNA is

$$f = 1 - (1 - e^{-t/\chi})^n$$

Examining Table I, we pick 0.1 as an *upper* limit for f, t = 70 s, and n = 2000 giving, as an *upper* limit, χ = 8 s and the first-order dissociation rate constant, which is $1/\chi$, equal to 0.125 s^{-1}.)

Despite the rapid dissociation of TF1-DNA complexes, a portion of originally bound TF1 sediments with SPO1 DNA (Figure 4), even during a 4-h centrifugation (Figure 5a). This is probably *not* due to tighter binding by a fraction of TF1 molecules to SPO1 DNA, but results from frequently repeated dissociation and reassociation of TF1 and DNA within the sedimenting zone. In the presence of competing, unlabeled, TF1, reassociation results in isotope exchange so that dissociation of labeled TF1 is complete (Figure 5c). The sedimentation analyses (which are marred by incomplete recovery of TF1, but not DNA, from the gradients) show that TF1-DNA complexes sediment considerably more rapidly than DNA, only when many molecules of TF1 are bound. The increase in sedimentation rate is out of proportion to the TF1-associated increase in mass. We do not know whether a kinking or flexing of the DNA is responsible for the increased sedimentation rate or whether intermolecular aggregates form.

The Affinity of TF1 for SPO1 DNA. We have used three sedimentation methods all of which could, in principle, be used to measure TF1-DNA binding equilibra: (1) sedimentation of TF1-DNA complexes out of a supernatant zone into a conventional sucrose gradient (Figure 4), (2) sedimentation of DNA through TF1 (Figure 6), and (3) short-column sedimentation (Figures 7-12).

In the first of these methods, the bound TF1 dissociates and it is difficult to quantitate displaced TF1. Nevertheless, the results of Figure 4 are qualitatively in accord with the detailed binding measurements that follow. If one assumes all of the displaced TF1 (Figure 4b, curve 1) to have been bound to SPO1 DNA in the supernatant zone, one can calculate an association constant, assuming a simple equilibrium of monovalent ligand with noninteracting sites (but see below). Using a value of 0.65 μg/μg of DNA for the capacity of SPO1 DNA for TF1 (Figure 6), and assuming all free TF1 to have been recovered (legend of Figure 4a), one calculates K_a = 4.4 mL/μg. This is within a factor of three higher than the more precise value calculated below for a slightly different solvent; the discrepancy is probably principally due to the uncertainty regarding TF1 recovery from the gradient.

The second method, which is relatively laborious and which consumes much labeled TF1, was only used to measure the capacity of SPO1 DNA for TF1 (Figure 6). The short-column centrifugation method was instead worked out (Figure 7) and used for a detailed analysis of TF1-DNA binding at 20 °C in buffer PK, ionic strength 0.175. The binding data (Figure 10) fit poorly to a Langmuir isotherm, that is, to a model of monovalent ligand binding to isolated, noninteracting, sites (Figure 10, curve 1). The deviations, which are made more obvious by transformation to a Scatchard plot (Figure 12), are consistent with two kinds of interpretation.

The first of these interpretations invokes an additional equilibrium between active and inactive TF1. TF1 is evidently composed of two identical polypeptide chains. If the monomer of TF1 was inactive in DNA binding (more precisely, if K_a for the monomer-DNA interaction were at least tenfold lower than for the dimer-DNA interaction), one would have to consider both the dimerization and the binding equilibra

$$2 \text{ monomer} \rightleftarrows \text{TF1} \ (K_2)$$

$$\text{TF1} + \text{DNA} \rightleftarrows \text{TF1-DNA} \ (K_a)$$

with K_2 and K_a as their respective association constants. The data of Figure 10 are adequately fitted by K_2, K_a pairs in the range of K_2 = 0.1 mL/μg, K_a = 9.1 mL/μg (Figure 10, curve 2 and Figure 12, curve 2) to K_2 = 0.5 mL/μg, K_a = 4.3 mL/μg (Figure 12, curve 3). The temperature dependence of binding seen in Figure 11 might also be explained as arising from a balance between tighter binding and greater dissociation of TF1 at elevated temperature. On the other hand, although we have not examined TF1 dissociation in detail, there is no evidence that TF1 does dissociate as readily as implied by these values of K_2 (i.e., 50% dissociation at 20 μg/mL for K_2 = 0.1 mL/μg; Johnson and Geiduschek, 1972).

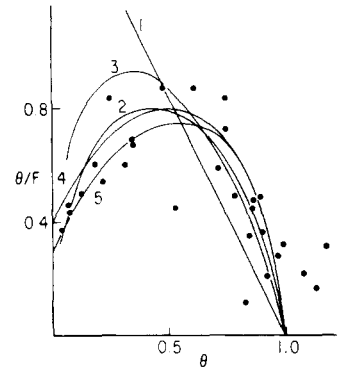

FIGURE 12: Scatchard plot of the data of Figure 10. F is the concentration of all species of unbound TF1 (μg/mL). θ is the fractional saturation of SPO1 DNA with TF1, taking the capacity to be 0.58 μg of TF1/μg of DNA. Calculated curves: (1) Langmuir isotherm, K_a = 1.7 mL/μg; (2) TF1 monomer-dimer equilibrium, K_2 = 0.1 mL/μg, K_a = 9.1 mL/μg. (3) TF1 monomer-dimer equilibrium, K_2 = 0.5 mL/μg, K_a = 4.3 mL/μg; (4) cooperative binding (McGhee and von Hippel (1974, 1976) eq 15 with n = 1) ω = 4, ω^2K = 6.4 mL/μg; (5) cooperative binding, ω = 5, ω^2K = 7.5 mL/μg.

Alternatively and more probably, TF1 binding to DNA is moderately cooperative. In terms of models for ligands on an infinite linear lattice (e.g., McGhee and von Hippel, 1974), the data can be adequately fitted by association constants for isolated ligands, K_a = 0.4-0.3 mL/μg, and cooperativity parameters, ω = 4-5, respectively (Figure 10, curves 3 and 4; Figure 12, curves 4 and 5), using a value of 0.58 μg of TF1/μg of SPO1 DNA for the binding capacity. The measurement of the capacity is the least satisfactory aspect of the experiment. At high TF1 concentrations, relatively small variations in the blank)loss of TF1 from the supernatant during centrifugation without DNA) lead to substantial variations in estimates of binding. Since the binding isotherm is not a Langmuir isotherm and is not known a priori, the capacity cannot be derived unequivocally from binding data at partial saturation. Therefore, it is not certain that the plateau of TF1 binding has been reached at the highest TF1 concentrations in Figure 10. Nevertheless, the above value of the binding capacity is in reasonably good agreement with the value derived in Figure 6 (in a medium of comparable ionic strength).

The significance of these values of the cooperativity parameter, ω, is that TF1 molecules preferentially bind in clusters. TF1 molecules bound in the interior of clusters have an association constant of ω^2K_a = 7.5-6.4 mL/μg; their standard free energy for binding to SPO1 DNA is more negative than

that of isolated ligands by 2 RT ln ω or 1.6–1.9 kcal/mol TF1 for ω = 4–5. Of course, these numbers apply only to a particular model, of cooperating ligands binding in linear arrays with nearest-neighbor interactions. It is not certain that TF1 binds to DNA in this way. A capacity, n, of only 0.6 μg/μg, which is one TF1 molecule per 60 nucleotide pairs or per 200 Å of DNA, implies that an SPO1 DNA molecule cannot bind TF1 along its entire length. If binding sites for clusters of TF1 molecules are discrete and separate, then TF1 molecules may not bind to these sites as linear arrays. Alternative possibilities would include rings or helical arrays of TF1 molecules around the DNA helix or oligomeric cores of TF1 molecules surrounded by DNA (Rill and Van Holde, 1973; Hewish and Burgoyne, 1973; Noll, 1974; Olins and Olins, 1974; Kornberg and Thomas, 1974; Baldwin et al., 1975; Griffith, 1976).

Cooperativity can also arise as a result of an unwinding of the DNA by the protein ligand, since DNA unwinding is itself cooperative. The comparable affinity of helical and denatured SPO1 DNA (Figure 9b and Results, section 1) argues against this interpretation.

TF1 was originally isolated and named in terms of its selective effect on the transcription of phage DNA. Yet, it closely resembles, in its amino acid composition and size, proteins that now appear to occur widely among the procaryotes. Searcy (1975), Rouvière-Yaniv and Gros (1975), and Haselkorn and Rouvière-Yaniv (1976) have purified such small, basic, proteins from *E. coli*, from cyanobacteria, and from a thermophilic mycoplasma. The *E. coli* protein HU and the cyanobacterial protein cross-react antigenically. These proteins and TF1 have strikingly similar amino acid compositions (Johnson and Geiduschek, 1972; Searcy, 1975; Rouvière-Yaniv and Gros, 1975; Haselkorn and Rouvière-Yaniv, 1976). Like the histones, these four procaryotic proteins lack cysteine and tryptophan. They either contain very little or no tyrosine and little or no methionine. The closely related *E. coli* HU and cyanobacterial proteins contain some histidine, while the "histone-like" thermoplasma protein and TF1 contain none. The *E. coli* and cyanobacterial proteins contain significantly less glutamine and glutamic acid than the other two proteins, and TF1 contains less arginine than the other three. The repressor coded by the phage λ *cro* gene has very recently been purified (Folkmanis et al., 1976) and has been shown to be a dimer of basic subunits having a molecular weight of approximately 9500. The amino acid compositions of the *cro* repressor and of the *E. coli* HU protein are strikingly similar (Y. Takeda, A. Folkmanis, and H. Echols, personal communication, to be published). The thermoplasma histone-like protein and TF1 have chains containing 102 and 106 amino acids, respectively. HU and the cyanobacterial proteins are slightly smaller, and the *cro* repressor is the smallest of the five. These proteins are closest in size to the smallest of the histones, histone H4, but their amino acid composition more closely resembles that of the larger histone H2b. Despite their slightly varying amino acid composition, one would judge that these proteins belong to a single ubiquitous class of procaryotic proteins. It is most interesting that the five proteins which have been identified thus far exhibit somewhat different properties. Only TF1 and the *cro* repressor are known to bind selectively to a particular DNA. The *cro* protein exhibits some selective affinity for a particular nucleotide sequence (Folkmanis et al., 1976), while TF1 is here shown to bind at many sites to SPO1 DNA, which contains a special nucleotide, hydroxymethyluracil. The *E. coli* HU protein nonselectively increases transcription of λ DNA (Rouvière-Yaniv and Gros, 1975), while the *cro* protein is a repressor (Folkmanis et al., 1976) and TF1 selectively inhibits transcription of hydroxymethyluracil-containing DNA (Wilson and Geiduschek, 1969). It seems likely that the exploration of this class of bacterial proteins will provide a fresh perspective on the interrelations of structure, nucleic acid binding, and regulatory functions of proteins.

Acknowledgments

We are grateful to B. H. Zimm for a critical reading of the manuscript.

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Separation of Oligo(adenosine diphosphate ribose) Fractions with Various Chain Lengths and Terminal Structures†

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ABSTRACT: Oligo(adenosine diphosphate ribose) preparations with chain lengths of 3 to 10 adenosine diphosphate ribose units were fractionated according to their chain lengths and their terminal structures by hydroxylapatite column chromatography and then polyacrylamide gel electrophoresis. The peak fractions from the hydroxylapatite column were each separated into two distinct subfractions by gel electrophoresis. The two subfractions were found to differ in chain length and terminal structure. A linear correlation was observed between the mobility and the logarithm of the chain length of oligo(adenosine diphosphate ribose) on gel electrophoresis, irrespective of the terminal structure.

Poly(ADP-Rib)[1] can be synthesized from NAD using isolated nuclei or chromatin of mammalian cells (Chambon et al., 1966; Fujimura et al., 1967; Nishizuka et al., 1967; Sugimura, 1973). ADP-Rib moieties from NAD are linked repeatedly by ribose–ribose (1′-2′) glycosidic bonds to form poly(ADP-Rib). Poly(ADP-Rib) is suggested to bind covalently to histone (Nishizuka et al., 1968; Otake et al., 1969; Smith and Stocken, 1975) and to have a regulatory role in DNA synthesis and mitosis (Burzio and Koide, 1970; Smulson et al., 1971; Miwa et al., 1973).

ADP-ribosylation of protein gives rise to a polymer in which the ADP-Rib units are repeated 1–20 times. In the in vitro reaction, DNA and histone increase both the chain length and the number of poly(ADP-Rib) chains (Yamada and Sugimura, 1973). Addition of DNase I to the system for poly(ADP-Rib) synthesis in vitro increases the number and the chain length of poly(ADP-Rib) formed (Miller, 1975). This paper reports the separation of oligo(ADP-Rib)[2] fractions of various chain lengths and different terminal structures, using hydroxylapatite column chromatography and then polyacrylamide gel electrophoresis. The elution profiles on chromatography and on electrophoresis were reproducible. Hydroxylapatite column chromatography and/or polyacrylamide gel electrophoresis can be used to determine the distribution of oligo(ADP-Rib) fractions of various chain lengths, and to isolate oligo(ADP-Rib) of a desired chain length.

Materials and Methods

Chemicals and Enzymes. NMN, ATP, and *Escherichia coli* alkaline phosphomonoesterase (EC 3.1.3.1) were purchased from Sigma Chemicals Co., St. Louis, Mo. Deoxyribonuclease I (EC 3.1.4.5), pancreatic ribonuclease (EC 3.1.4.22), and snake venom phosphodiesterase (EC 3.1.4.1) were obtained from Worthington Biochemical Corp., Freehold, N.J. Snake venom phosphodiesterase was purified to remove 5′-nucleotidase (EC 3.1.3.5), by the procedure of Sulkowski and Laskowski (1971). Pronase E was purchased from Kaken Chemicals, Tokyo. [*adenine*-8-^{14}C]ATP (47 mCi/mmol) was a product of Schwarz/Mann, Orangeburg, N.Y. Acrylamide and *N,N′*-methylenebisacrylamide were products of Wako Pure Chemical Industries, Ltd., and Seikagaku Kogyo Co., Ltd., Tokyo, respectively. Temed was from Eastman Kodak Co., Rochester, N.Y., and Bio-Gel P-2 and Bio-Gel HTP were from Bio-Rad Laboratories, Richmond, Calif.

Preparation of Oligo(ADP-Rib). Oligo(ADP-Rib) and poly(ADP-Rib) were prepared from [*adenine*-8-^{14}C]ATP and nicotinamide mononucleotide using isolated rat liver nuclei, and purified as described by Sugimura et al. (1971), with the following modification in order to preserve oligo(ADP-Rib). The aqueous layer obtained after the second phenol extraction was subjected to gel filtration on a Bio-Gel P-2 column (2 × 40 cm) equilibrated with 1 mM sodium phosphate buffer (pH 6.8). Oligo(ADP-Rib) was separated from poly(ADP-Rib) by hydroxylapatite column chromatography as described in the following paragraph.

Hydroxylapatite Column Chromatography. Bio-Gel HTP was suspended in 1 mM sodium phosphate buffer (pH 6.8) and packed in a column (1 × 10 cm). The sample (300–400 OD_{260}

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[1] The abbreviations used are: ADP-Rib, adenosine diphosphate ribose; oligo(ADP-Rib), oligo(adenosine diphosphate ribose); poly(ADP-Rib), poly(adenosine diphosphate ribose); Ado(P)-Rib-P, 2′-(5″-phosphoribosyl)-5′-AMP; Ado(P)-Rib, 2′-(ribosyl)-5′-AMP; Ado-Rib-P, 2′-(5″-phosphoribosyl)adenosine; Rib-P, 5′-phosphorylribose; BPB, bromophenol blue; Temed, *N,N,N′,N′*-tetramethylethylenediamine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

[2] Poly(ADP-Rib) is here defined as polymer containing more than 11 ADP-Rib units and oligo(ADP-Rib) as polymer containing 2 to 10 ADP-Rib units.