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DNA Unwinding Protein from Meiotic Cells of *Lilium*†

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ABSTRACT: An ATP-dependent DNA unwinding protein is present at a high level of activity in meiotic cells of lilies. The protein also acts as a DNA-dependent ATPase, the single strand form being the preferred cofactor. It binds in the absence of ATP to single-strand DNA and to ends or nicks in duplex DNA. A 3′-OH terminus is required for binding at duplex ends; such binding is highly stable. Unwinding occurs in the presence of ATP, and it is limited to about 50 base pairs per end or 400–500 base pairs per nick. The ATP hydrolyzed during unwinding is distinguishable from ATP hydrolysis in the presence of single-strand DNA.

Several types of DNA unwinding proteins which require ATP for activity have been prepared from *Escherichia coli* (Abdel-Monem & Hoffmann-Berling, 1976; Abdel-Monem et al., 1976, 1977a,b; Richet & Kohiyama, 1976). The proteins bind to ss-DNA[1] (Abdel-Monem & Hoffmann-Berling, 1976) and, when so bound, function as ATPases. The biological role of the ATPase activity is unknown, but the activity has been useful as an assay in purifying proteins of this kind. By using the assay, we have identified and partially purified a DNA unwinding protein ("U-protein") in meiotic cells of *Lilium.* Among the four chromatographically separable DNA-dependent ATPase activities present in meiotic cells, only U-protein increases markedly during meiosis. As will be discussed fully in a separate study, the increase coincides with the pairing and crossing-over of the chromosomes and is low during premeiotic S phase. Its behavior thus contrasts with the behavior of the *E. coli* "rep protein" which has been assigned a specific unwinding role in ΦX-174 replication (Scott et al., 1977). Although U-proteins are probably involved in DNA replication of higher eukaryotes, this paper is confined to the meiotic U-protein which, because of its prominence during meiotic prophase, is probably involved in recombination.

In general, the U-protein of lily meiocytes is similar in its properties to those of the *E. coli* U-proteins I and II described by Hoffmann-Berling and his collaborators (Abdel-Monem et al., 1977c). There are, nevertheless, several differences in property between the *E. coli* and *Lilium* proteins which may be related to their respective biological roles. The *E. coli* U-proteins I and II do not bind to duplex DNA and require an appreciable but still undetermined length of ss-DNA to unwind an adjacent duplex region (Abdel-Monem et al., 1977a,b). Lily U-protein can bind to duplex DNA and can initiate unwinding from the ends of linear molecules, or from nicks in linear and circular forms. Except for very short DNa duplexes (less than 100 bp), the lily U-protein does not fully unwind ds-DNA; it initiates and effects unwinding over an apparently fixed length of DNA from the point of initiation.

Materials and Methods

1. Isolation of Cells. Meiotic cells ("microsporocytes") were extruded from anthers by squeezing individual flower buds of

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[1] Abbreviations used: ss-DNA, single stranded DNA; ds-DNA, double stranded DNA; bp, base pairs.

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appropriate size (Stern & Hotta, 1970). Bulk homogenization of flower buds was unproductive because the phenolic compounds in the outer portions of the buds produced an extreme blackening of extracts; in any case, meiotic cells are very difficult to purify from such homogenates. The material extruded from buds consisted largely of microsporocytes, and they were collected in ice-cold White's culture medium. After three washings, they were suspended in a glycerin–sucrose solution consisting of 0.5 M sucrose–50% (v/v) glycerol–0.01 M Tris-HCl (pH 7.2)–1 mM $CaCl_2$–3 mM $MgCl_2$–10 mM KCl (Hotta & Stern, 1965). When thus suspended, the microsporocytes could be stored at −20 °C for a period of at least 9 months without significant loss in activity of the protein. Most of the material used in these studies (about 50 000 buds) was collected during the summer from commercial plots at the Oregon Bulb Farms (Gresham, Oregon).

2. Enzyme Extraction. Preparations of enzyme were usually made with 10 mL of packed meiotic cells obtained from approximately 1000 flower buds. A few preparations were made with 50–100 mL of cells. Regardless of amount, the cells, which contained about 50 mg of total protein per mL of packed volume, were homogenized with a glass tissue grinder in a solution of 0.01 M Tris-HCl (pH 8.5)–1 mM EDTA–0.1 mM dithiothreitol, and centrifuged at 10 000*g* for 10 min to remove large debris. The remainder was clarified by centrifuging at 100 000*g* for an additional 30 min. The supernatant fluid was 30% saturated with ammonium sulfate and the precipitate discarded. The ammonium sulfate concentration was increased to 45% saturation and the precipitate collected by centrifugation. It was dissolved in 0.01 M Tris-HCl (pH 8.1)–0.1 mM dithiothreitol (Tris–dithiothreitol), using 1 mL of solution per 2 mL of initial packed cell volume, and the mixture dialyzed for 6 h or longer against Tris–dithiothreitol. The clear dialyzed solution was applied to a 1 × 3 cm column of denatured DNA-cellulose (Alberts & Herrick, 1971) and eluted with a linear gradient of NaCl generated from 75 mL of Tris–dithiothreitol and 75 mL of 0.8 M NaCl in Tris–dithiothreitol. The peak of ATPase activity eluted at 0.45–0.5 M NaCl. About 12–18 mL of eluate from the peak region was pooled, dialyzed overnight against Tris–dithiothreitol (pH, 7.5), concentrated to about 1–2 mL by further dialysis against 40% (w/v) poly(ethylene glycol) (6000–7500) in Tris–dithiothreitol, and rerun through the DNA-cellulose column. The tubes with peak activity were processed as before. For storage, the concentrated preparation was diluted with an equal volume of glycerol and kept at −20 °C. Usually, the solution contained 0.25–1.0 mg of protein/mL and could be stored in this way for at least 6 months without any loss of activity.

3. ATPase Assay. Samples were incubated for 15 min at 35 °C in 0.25 or 0.5 mL of medium which consisted of 50 μM [γ-^{32}P]ATP (2×10^5 cpm/μmol)–5 mM $MgCl_2$–0.1 mM dithiothreitol–50 μg/mL of heat denatured calf thymus or lily DNA. The calf thymus was a commercial product (Calbiochem. Calif.); DNA from *Lilium* was prepared as previously described (Hotta & Bassel, 1965). The reaction was stopped by quick cooling and addition of 0.5 mL of charcoal suspension (5 g of activated charcoal in 100 mL of 0.01 M Tris-HCl, pH 7.5). The suspension was centrifuged for 2 min at 3000 rpm in an International PR-6 centrifuge and the radioactivity in the supernatant fluid determined. The radioactivity thus measured was considered to be due to inorganic ^{32}P derived from ATP and is expressed as μmol of ATP hydrolyzed in 15 min.

The products of enzymatic hydrolysis were identified by using [2,8-^{3}H]ATP as substrate. The reaction was stopped by addition of 2 volumes of 95% ethanol and the mixture centrifuged. The supernatant fluid was concentrated under vacuum and 20 μg each of ADP and AMP were added to the sample which was then spotted on Whatman No. 1 filter paper. A descending chromatogram was developed using isoamyl alcohol saturated with aqueous 5% Na_2HPO_4 (Wyatt, 1955). Spots containing tri-, di-, and monophosphates of adenosine were cut out and eluted for counting. After 15 min of incubation, the respective counts in ATP, ADP, and AMP were 45 000, 4370, and 95: after 30 min, they were 39 300, 9830, and 125. Unincubated controls had activities of 40–100 cpm in ADP and AMP spots. We consider the AMP radioactivity in the incubated samples to be negligible and conclude that ADP and P_i are the products of U-protein mediated hydrolysis.

4. Nuclease Assays. (a) Exo- plus endonuclease activity was measured at 35 °C in 0.2 mL of medium containing 0.65 μg of *E. coli* [^{3}H]DNA (8.5×10^4 cpm)–5 mM $MgCl_2$–10 mM Tris-HCl (pH 7.5)–0.1 mM dithiothreitol–0.1 mM ATP–50 μg of U-protein. The incubation was stopped after 30 min by addition of 100 μg of carrier DNA and of trichloroacetic acid to a final concentration of 10% (w/v). Radioactivities were measured in the acid soluble and insoluble fractions.

(b) Endonuclease activity was measured under the same conditions of incubation as in a except for the use of 0.5 μg of T7 [^{3}H]DNA (10 000 cpm) as substrate and the doubling of the amount of U-protein to 100 μg per assay. Incubations were terminated by cooling and adding EDTA and NaOH to final concentrations of 5 mM and 0.3 N, respectively. Each mixture was layered over an alkaline glycerol gradient and centrifuged at 30 000 rpm for 12 h in an SW40.1 Beckman rotor (Hotta & Stern, 1974). Any significant change in sedimentation profile would have been attributed to endonuclease activity but none was found.

(c) Phosphatase activity was measured with partially digested *E. coli* [^{32}P]DNA as substrate. The DNA (0.4 mg) was dissolved in 0.5 mL of 0.01 M Tris-HCl (pH 7.5)–5 mM $MgCl_2$–1 mM $CaCl_2$. Ten units of micrococcal nuclease (Worthington) was added and the mixture incubated for 5 min at 37 °C. The deproteinized product was used as substrate for 3′-phosphatase activity. For a 5′-phosphatase substrate, 1 μg of DNase I was used and the mixture incubated for 5 min at 20 °C. Twenty micrograms of treated DNA (50 000 cpm/μg) in 50 μL was added to 250 μL of assay medium (Tris–dithiothreitol–5 mM $MgCl_2$) containing 50 μg of U-protein, and the mixture incubated at 35 °C. Samples were taken at 5- or 10-min intervals up to 40 min for measurement of inorganic ^{32}P as in the ATPase assay. Where activity was detectable, it was linear for at least 40 min.

5. DNA Binding. (a) DNA Substrates. Several types of DNA were used as substrates for binding measurements. ΦX-174 DNA (^{3}H ≈ 5000 cpm/μg, kindly provided by Dr. M. Hayashi) was prepared in closed circular (RF I), open circular (RF II), and single-stranded forms (Hayashi & Hayashi, 1971). T7 DNA (^{3}H ≈ 6000 cpm/μg), prepared according to the procedure of Thomas & Abelson (1966), served as a source of linear duplex DNA. Relatively short lengths of duplex DNA were prepared by treating isolated lily nuclei with micrococcal nuclease and the nucleosomes thus formed deproteinized to yield duplex DNA fragments in the range of 100–800 bp (^{3}H ≈ 3000 cpm/μg). Generally, 1 mL of nuclear suspension containing 100 μg of DNA was incubated with 300 units of the enzyme for 10 min at 30 °C, and the DNA was then extracted by a standard procedure (Hotta & Bassel, 1965). The purified DNA fragments were suspended at a concentration of 200 μg/mL, layered over a 10–30% glycerol gradient containing 0.01 M Tris-HCl (pH 7.5)–0.1 M NaCl–1 mM EDTA, and

centrifuged at 25 000 rpm in a Beckman SW 27.1 rotor for 48 h. Fractions were collected from the bottom of the tube and pooled according to fragment size. Each of the pooled fractions was layered over a fresh glycerol gradient and recentrifuged. Fractions from the appropriate region of the gradient were collected as before and pooled. The DNA was treated with 5 μg/mL of alkaline phosphatase for 10 min at 35 °C, deproteinized, dialyzed overnight against distilled water, and lyophilized. Fragment sizes were checked by electrophoresis in a 2.5% agarose gel (Kornberg, 1974) using *Hin*d II fragments of ΦX-174 DNA as markers.

(b) Binding Assay. The standard medium for measuring DNA-protein binding consisted of 0.01 M Tris-HCl (pH 7.5)-2 mM $MgCl_2$-0.1 mM dithiothreitol (Tris-Mg-dithiothreitol) and 1 mM ATP where indicated. Each assay mixture was 0.25 mL and was incubated for 15 min at 35 °C. The mixture was layered over a 10-30% glycerol gradient containing 0.1 M NaCl in Tris-Mg-dithiothreitol. For sizes in the range of intact or nicked T7 DNA, tubes were centrifuged at 30 000 rpm for 8 h at 5 °C in an SW40.1 rotor (Beckman). The speed was increased to 33 000 rpm for ΦX DNA. In the case of 100-800-bp fragments, samples were spun for 18 h at 40 000 rpm in an SW50.1 rotor. Fractions were collected from the bottom of the tubes. One aliquot from each fraction was used for measuring radioactivity, and the other for DNA-dependent ATPase.

6. DNA Unwinding. The assay medium was identical with the one used in DNA binding; ATP was always present. The reaction was stopped by adding 3 volumes of 0.01 M sodium acetate (pH 4.7)-10 mM $ZnCl_2$-200 units/mL of S1 nuclease, and incubated for 30 min at 37 °C. Under these conditions single-stranded DNA is digested to acid-soluble form even in the presence of saturating amounts of U-protein. After digestion, samples were chilled and supplemented with 0.1 M EDTA, pH 9.0, and 100 μg of carrier DNA. Cl_3CCOOH was added to a concentration of 10% (w/v) for DNA precipitation. The precipitates were collected on glass filter discs and the radioactivity was determined. In somes cases, samples of filtrates were collected dropwise on Whatman GF/C papers with intermittent drying in order to determine acid-soluble counts directly.

7. DNA-RNA Unwinding. ΦX-174 RNA-DNA hybrids were provided by Dr. M. Hayashi. These were synthesized in vitro using DNA "+" strands by RNA polymerase, and the four NTPs including [^{3}H]CTP (Hayashi & Hayashi, 1971). The ratio of RNA/DNA in the hybrid was approximately 0.8. Release of RNA from DNA was assayed in three different ways: (1) The incubation mixture was layered over 10-30% glycerol gradient and the radioactivity profile traced after centrifugation at 33 000 rpm in an SW40.1 rotor for 8 h. [^{3}H]RNA released from the hybrid sedimented over a range of 5-10 S, whereas the hybridized form sedimented at 13 S. (2) The incubation mixture was diluted with Tris-dithiothreitol (pH 7.5) and NaCl added to a final concentration of 0.6 M. The mixture was passed through a filter and the adsorbed radioactivity determined. Hybridized RNA in the presence of U-protein was retained by the filter; unhybridized RNA was not. (3) RNase A was added to the incubation mixture to a concentration of 2 μg/mL and the acid-soluble radioactivity was measured. Free RNA in the presence of U-protein is totally digested under these conditions.

Results

Purification of U-Protein. The procedure used was similar to the one described by Abdel-Monem & Hoffmann-Berling (1976). The course of protein purification is summarized in Table I. The concentration of U-protein in the crude homogenate could not be estimated because of the very high nonspecific ATPase activity. The nuclear fraction, and in particular its lipoprotein subfraction which contains the DNA-binding "R-protein" (Hotta & Stern, 1971; Mather & Hotta, 1977), could be assayed for U-protein content because of the low nonspecific ATPase activity, but DNA-dependent ATPase activity was not found. The U-protein could only be detected in the soluble cell fraction after partial purification. It is possible that the U-protein, like other soluble proteins in nuclei, was lost by leakage in the course of fractionation, but other possibilities, such as a masking of activity, cannot be excluded.

TABLE I: Stages in Purification of U-Protein from *Lilium*.

Fraction	Protein content (mg)	DNA-dependent ATPase (nmol of ATP/μg of protein)
Homogenate	1750	
Supernate (100 000*g*)	199	
30-45% Satd $(NH_4)_2SO_4$	105	0.029
DNA-Cellulose eluate	0.128	17.8

The most effective step in the purification procedure is passage of the extract through a DNA-cellulose column after ammonium sulfate fractionation. Eighty percent of nonspecific ATPase activity is removed by use of ammonium sulfate. Less than 10% of the total ATPase activity adsorbed to the DNA column was nonspecific and it was present to a similar extent under the four peaks of DNA-dependent ATPase activity. The peaks eluted at 0.22, 0.35, 0.45, and 0.62 M NaCl, the major one coming off at 0.46 M. When this peak was rerun on DNA-cellulose, the nonspecific ATPase activity was at the limit of detection. Most of the characterizations reported here were done on preparations carried to this point of purification. It will be seen that the degree of purification achieved is satisfactory for the analysis reported here.

In assessing the purity of the preparation we were mainly concerned with the relationship between ss- and ds-DNA binding, ATP hydrolysis, DNA unwinding at nicked regions, and DNA unwinding at duplex ends. These properties, which are considered in detail later, could belong to different proteins that copurify because of their common affinity for ss-DNA. As a preliminary to their detailed characterization we report four analyses bearing on the comparative purity of the preparations used. Figure 1 shows a profile of the three principal enzymatic activities obtained by running one of the preparations through a Sepharose 6B column. The profiles of ATPase, nick unwinding, and end unwinding are entirely superimposable. Figure 2A shows the acrylamide gel pattern of an undenatured preparation. A single strong band is evident and, although minor contaminants may be present, assays of the three enzyme activities carried out on a companion gel divided into 2-mm slices showed the three activities to be virtually identical in distribution and to coincide with the position of the protein band (data not shown). The denaturing gel (Figure 2B) also shows a single protein band. Thus, neither gel filtration, electrophoresis of native and denatured preparations, nor zonal sedimentation (Figure 3) effected a separation of activities or resolved the preparation into multiple protein components. Similarly, as will be indicated for the data in Figure 5, the protein binding to nicks or ends of DNA duplexes retains all the characteristics assigned to the U-protein. These results lead us to infer that the characteristics studied are most likely the

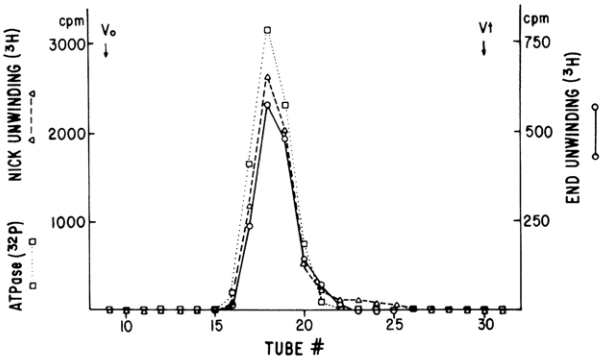

FIGURE 1: Elution profile of U-protein activities from a Sepharose-6B column (0.8 × 30 cm). The Sepharose was suspended in and washed with 10 mM Tris buffer (pH 7.5)–0.2 mM dithiothreitol–1 mM $MgCl_2$–10% (v/v) glycerin. One hundred micrograms of DNA-cellulose purfied protein dissolved in 0.2 mL of buffer was loaded on the column and eluted with the glycerin–Tris buffer in 0.1-mL fractions. Portions (0.1 mL) were used for each of the assays. Detailed descriptions of the activities plotted (ATPase, nick unwinding, and end unwinding) are provided further on in the text. All activities are expressed per 0.1 mL of eluate: ATPase, cpm of inorganic ^{32}P released in 15 min at 35 °C; nick and end unwinding, cpm of [^{3}H]DNA rendered S1 digestible in 30 min at 35 °C.

properties of a single protein or of a tightly associated protein complex.

General Characteristics of U-Protein: Physical. The molecular weight of the purified protein was estimated to be 130 000 by Sephadex filtration. It sedimented in glycerol gradients over a wide range of NaCl concentrations with a $s_{20,w}$ of 8 S (Figure 3). Except for the small peak at 14 S, and in some cases an equally small one at 20 S, no aggregation or disaggregation of activities was observed between 0.1 and 0.7 M NaCl. Above 0.7 M NaCl, minor peaks were not observed.

Enzymatic. Hydrolysis of ATP to ADP required the presence of DNA. Experiments on cofactor specificity are summarized in Table II. ss-DNA, whether linear or circular, is much more effective than ds-DNA, but the source of DNA is of minor consequence. Supercoiled DNA (RF I) is virtually inactive as a cofactor, but the open circular form (RF II) is comparable in activity with ds-DNA from T7 or *Lilium*. Free ends or nicks appear to be essential for ds-DNA activity. Synthetic polydeoxynucleotides were generally ineffective. Some RNA preparations functioned as cofactors but even the most active one (mRNA, *Lilium*) was within the range of ds-DNA. The high activity of the RNA–DNA hybrid may be due to the ss-DNA between the hybrid regions; unwinding of the hybrid was not observed (Table III).

Nuclease activities were measured in presence and absence of ATP (Materials and Methods, sections 4a,b). Significant levels of exo- or endonuclease activities could not be detected after 30-min incubation with 50–100 μg of protein. Phosphatase activities (Materials and Methods, section 4c) were either at or near the limits of detection. Approximately 0.7 pmol of 5′-phosphoryl residues were released in 30 min per μg of protein. Levels of 5 pmol per μg of protein were found in case of 3′-phosphoryl residues, but only in absence of Mg^{2+}. If present at concentrations used in unwinding assays, 3′-phosphatase activity was at the limit of detection.

DNA Binding. Binding to DNA was analyzed by zonal sedimentation and DNA–protein complexes were identified by the position of [^{3}H]DNA and ATPase activity in the glycerol gradient (Figures 4 and 5). Whenever tested, the complexes formed had the capacity to unwind DNA in presence of ATP (data not shown). The interactions to be described thus

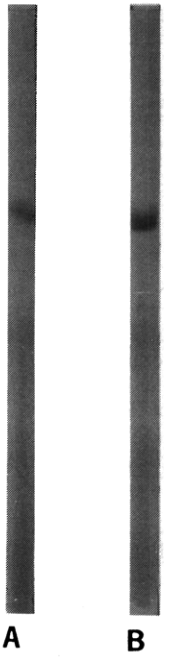

FIGURE 2: Disc gel electrophoresis of native and denatured U-protein preparations. Samples were loaded on cylindrical gels, 13 × 0.65 cm, consisting of 7% acrylamide (acrylamide:bisacrylamide = 2.01) in Tris–glycine buffer, pH 8.3. Gel B also contained 0.2% sodium dodecyl sulfate. Samples were run for 2 h at 100 V, 4 mA per gel. Gel A was loaded with 50 μg of protein from fraction obtained after a second passage through DNA-cellulose. Gel B was loaded with 50 μg of protein from fraction obtained after purification on Sepharose B column. Coomassie Blue was used to locate the protein. Both native and denatured protein preparations showed only one band in the gels. A duplicate of gel A was sliced into 65 portions and assayed for enzyme activities.

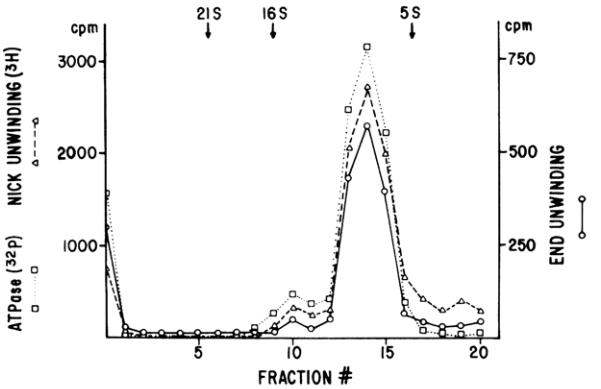

FIGURE 3: Zonal sedimentation of U-protein activities. A 0.2-mL sample containing 100 μg of protein purified through the DNA cellulose step was used in this analysis. The protein was dissolved in a solution of 10 mM Tris (pH 7.5)–0.2 mM dithiothreitol–1.0 mM $MgCl_2$ and layered over a 10–30% glycerin gradient containing the same buffer. The sample was spun for 4 h at 45 000 rpm, in an SW 50.1 rotor (Beckman) maintained at 4 °C. Fractions (0.25 mL) were collected from the bottom of the tube and 0.05 mL of eluate was used for each of the assays. Activities are expressed as in Figure 2. [^{3}H]DNA was used in each marker: 200 base pair fragments in lily DNA (5 S); RF II (16 S); RF I (21 S). The markers were centrifuged in separate tubes.

not only represent those between DNA and ATPase activity, but also between DNA and unwinding activity. Binding to ss-DNA occurred as expected (Figure 4); from the standpoint of unwinding, however, it is binding to ds-DNA that is of major interest.

Complex formation between U-protein and ds-DNA was difficult to detect with DNA molecules having more than 5000

TABLE II: Nucleic Acid Cofactors in ATP Hydrolysis by U-Protein of *Lilium*.[a]

Cofactor DNA source	Activity ds	Activity ss	Cofactor	Activity	Cofactor	Activity
Lilium	156	1180	Poly(dA)	61	rRNA *Lilium*	131
T7	204	1080	Poly(dC)	3	rRNA mouse	34
ΦX174-RFI	4		Poly(dG)	4	RNA VSV	105
-RFII	136		Poly(dT)	5	tRNA *Lilium*	37
-circular		304	Poly(dI)	40	tRNA mouse	11
-linear		928	Poly[d(A-T)$_n$]	41	mRNA *Lilium*	226
λ	96	1310	Poly[d(G-C)$_n$]	33	ΦX DNA-RNA	909

[a] Ten micrograms of nucleic acid and 30 μg of U-protein were used for each assay. The conditions of assay are described in the text. Activity is expressed in nmol of inorganic phosphate released in 30 min at 35 °C. The ΦX-174 DNA and the ΦX DNA-RNA hybrids were provided by Dr. Hayashi (Hayashi & Hayashi, 1971). RNA from vesicular stomatitis virus, Indiana strain ("VSV"), was a gift from Dr. John Holland. The synthetic polydeoxynucleotides were obtained commercially. Much of the activity in the DNA-RNA hybrid is attributable to the unhybridized DNA regions. λDNA was provided by Dr. M. Green. RNA was prepared by the method of Kirby (1968); rRNA and 4-5S RNA were separated on a sucrose gradient. mRNA was separated on an oligo(dT)-Sepharose column (Levenson & Marcu, 1976).

TABLE III: DNA Unwinding by U-Protein from *Lilium*.[a]

DNA substrate	S1-resistant radioact. (cpm) Original	−ATP	+ATP	DNA release (%) −ATP	+ATP	bp unwound/nick or end
Lilium: 800 bp	6 855	6 725	5 915	2	14	56
Lilium: 400 bp	6 855	6 540	4 880	5	29	58
Lilium: 200 bp	6 585	5 850	3 475	11	47	47
Lilium: 100 bp	6 875	5 485	1 995	20	71	36
Lilium: residue	6 445	4 885	400	24	94	
T7: intact	44 950	44 905	44 020	0.1	2	(400)
T7: nicked	4 090	4 010	2 720	2	33	560
ΦX 174: RFII	11 270	11 160	10 080	1	11	420
ΦX: DNA/RNA	1 596	1 506	1 505	6	6	

[a] Except for the DNA/RNA experiment, each reaction mixture contained 12 μg of DNA and 110 μg of lily U-protein. The standard unwinding assay was used. The columns under "S1 resistant . . ." represent the radioactivities in the original sample and those in the aliquots after incubation with U-protein in absence and presence of ATP. The last column contains the numbers of bp unwound at ends or nicks; these were calculated from the % DNA released in presence of ATP. In the case of *Lilium*, the spread of size of fragments was ignored and calculations assumed a single size of fragment for each of the preparations (the mean *S* value on a weight basis). The "residue" represents the duplexes remaining after S1 digestion of a preparation of 100 bp fragments that had been exposed to U-protein. Agarose gel measurements indicated the residue to contain duplexes in the range of 40–60 bp. They were deproteinized with chloroform:amyl alcohol and, after dialysis followed by lyophilization, they were assayed for unwinding. T7 DNA was prepared according to Thomas & Abelson (1966) and nicked with DNase I (1.5 μg/mL for 10 min at 20 °C); a sample of the product was analyzed in an alkaline glycerol gradient and the number of nicks per strand was estimated as 11.7. The ΦX RF II preparation was estimated as having an average of 1.4 nicks per molecule. The ΦX DNA/RNA hybrid was provided by Dr. M. Hayashi. DNA (0.5 μg) was present in the hybrid sample. It was prepared using an in vitro RNA synthesis system with the (+) strand as template and [^{3}H]CTP as source of label. The assay for unwinding is described under Materials and Methods, section 7.

bp. The contrast between ds- and ss-DNA is evident from the sedimentation profiles shown in Figure 4. Single-stranded T7 DNA which was pretreated with U-protein (Materials and Methods, section 5b) cosedimented with it; in contrast, the sedimentation behavior of T7 ds-DNA, similarly pretreated, was changed only slightly. The ATPase activity found in the ds-DNA region of the gradient was too small to be considered as evidence for the presence of U-protein. Supercoiled ΦX-174 DNA (RF I) was unaffected by U-protein, but there was a slight shift in the sedimentation profile of the nicked form (RF II). Although some questions can be raised about the significance of the small shifts observed with the duplex DNA of T7 and of ΦX-174 (RF II), it will be apparent from the experiments to be described that U-protein does bind to ends or nicks in duplex DNA.

Complex formation between U-protein and duplex DNA was tested with fragments of duplex DNA that were considerably smaller than T7 or ΦX molecules. The fragments, which were in the range of 100–400 bp (Materials and Methods, section 5a), were derived from lily DNA. These were assayed for binding in the same way as the larger DNA molecules. The results of the experiments are summarized in the sedimentation profiles in Figure 5. The small ss-DNA fragments behave like T7 ss-DNA. The small ds-DNA fragments, however, showed major shifts in their sedimentation profiles following incubation with U-protein. The shifts are not due to a specific affinity of lily U-protein for lily DNA: high molecular weight lily ds-DNA was indistinguishable from T7 ds-DNA with respect to complex formation. Moreover, the binding cannot be attributed to the presence of single-stranded tails in the duplexes from the micrococcal nuclease digests. Incubation of the duplex fragments with S1 nuclease prior to their exposure to U-protein had no effect on the previously observed shifts in sedimentation profiles of the DNA-protein complexes. We infer that the U-protein can bind at or near duplex ends and that such restricted binding would make it difficult to identify complex formation with large DNA molecules by zonal sedimentation analyses.

The combined results from the different analyses of binding activity permit two conclusions: (1) Binding does not require ATP. The sedimentation profiles of U-protein-DNA complexes shown in Figures 3 and 4 were obtained from mixtures that lacked ATP. We have not observed any significant change in the profiles by supplementing the mixtures with ATP. (2)

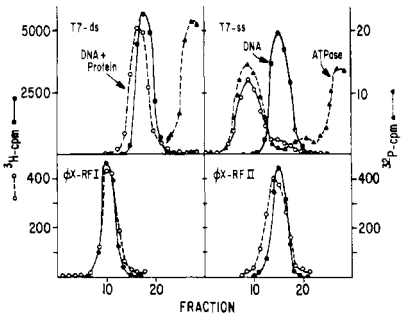

FIGURE 4: Zonal sedimentation patterns of mixtures of DNA and U-protein. Each profile represents 2 μg of DNA and 20 μg of U-protein. After incubation (see Materials and Methods, section 5b), the mixture was layered over a 10–30% glycerol gradient containing 0.01 M Tris (pH 7.5)–0.1 mM dithiothreitol–0.1 M NaCl–1 mM $MgCl_2$. The sedimentation profiles of protein-free ^{3}H-labeled ds- and ss-T7 DNA and of RF I and RF II ΦX-174 DNA are included as reference markers (closed circles). Profiles of [^{3}H]DNA incubated with U-protein are traced by the open circles. The dashed line is U-protein ATPase activity. The heavy end of the gradient is to the left.

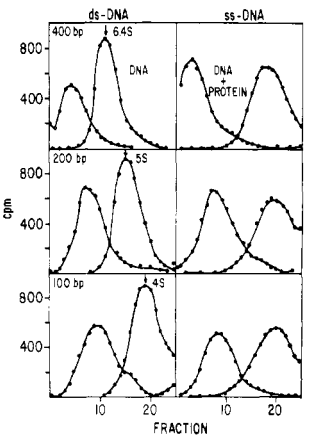

FIGURE 5: Binding of small fragments of ds- and ss-DNA from *Lilium* to U-protein. Fragments were products of micrococcal nuclease digestion (see Materials and Methods, section 5a). Procedure for sedimentation analysis is the same as in Figure 4. All samples of duplex DNA were treated with S1 nuclease and alkaline phosphatase prior to being mixed with protein. The direction of sedimentation is to the left.

U-protein binds more readily to ss- than to ds-DNA, probably because the binding with duplexes is restricted to end regions. Since it was more convenient to assay for unwinding than binding, several significant features of complex formation were examined by following unwinding behavior.

Unwinding Activity. The extent of duplex unwinding was determined by measuring the amount of S1-digestible DNA after its incubation with U-protein (Materials and Methods, section 6). Different forms and sizes of duplex DNA were tested and the results obtained are presented in Table III. The substrates fall into three groups, each of which displays distinctive unwinding behavior. (1) Lily DNA fragments show an increasing proportion of unwound duplex with decreasing size of fragment. Although the values are necessarily approximations because of the spread of sizes within each group, unwinding appears to be limited to about 50 bp per end. (2) Internally nicked duplexes of linear T7 or of circular ΦX-DNA are unwound to a much greater extent than are intact duplexes; for technical reasons (e.g., the presence of occasional nicks) the 2% value obtained for intact T7 DNA may not be a reliable measure of end unwinding. (3) DNA/RNA hybrids are not unwound to any significant extent by U-protein. Since no more than 80% of the circular DNA is hybridized (M. Hayashi, personal communication) and since the RNA involved has a mean length of about 700 bases (calculated from *S* values [Materials and Methods, section 6]), the hybrid duplex must have several single-stranded gaps. The absence of significant unwinding in the circular RNA–DNA duplex having multiple gaps contrasts with the behavior of the RF II sample which has an average of 1.4 gaps. Taken as a whole, the data in Table III indicate that U-protein can unwind DNA duplexes from ends or nicks and that it cannot unwind DNA/RNA hybrids. More detailed information has been obtained on the nature of the unwinding at duplex ends and on the relative extents of unwinding at ends and nicks.

The unwinding of lily DNA duplexes, which are presumably uninterrupted by nicks, does not depend upon the presence of single-stranded tails. As was the case in the binding experiments, treatment of fragments with S1 nuclease prior to their incubation with U-protein, had no effect on the degree of unwinding. However, fragments which are prepared by enzymatic treatment with micrococcal nuclease alone are very poor substrates for unwinding by U-protein. Less than 2% of 400 bp fragments thus prepared is unwound by U-protein regardless of whether ATP is present or absent in the incubation medium. To raise the level to the 47% shown in Table III requires prior treatment of the micrococcal digest with alkaline phosphatase. Corresponding results were obtained when sedimentation profiles were used to measure binding rather than unwinding. Neither binding nor unwinding occurs in micrococcal digestion fragments unless treated with alkaline phosphatase. Since micrococcal nuclease digestion yields fragments with 3′-phosphoryl termini, it appears likely that 3′-OH termini are required at duplex ends for both binding and unwinding. Nicking of T7 DNA with DNase I yields 5′-phosphoryl termini and such nicked molecules can be unwound by U-protein, the degree of unwinding being unaffected by phosphatase treatment. Moreover, incubation of phosphatase-treated micrococcal nuclease fragments or nicked T7 DNA with polynucleotide kinase to phosphorylate 5′-OH groups, had no effect on their susceptibility to unwinding. We conclude that 3′-OH groups are essential to unwinding at duplex ends and that nicks are indifferent to the presence or absence of phosphoryl groups at 5′ ends. As yet, we have been unable specifically to phosphorylate 3′-OH groups at sites of nicks.

The significance of the limited unwinding achieved at duplex ends in the absence of ATP (Table III) is unclear. The marginal level of such unwinding at nicked sites in T7 DNA has been repeatedly observed; whatever the reason, ATP-independent unwinding occurs only at duplex ends. On the other hand, the limited unwinding which occurs in the presence of ATP is general. The incompleteness of the reaction is demonstrable in several ways. Unwinding is not detected if the U-protein is removed from the complex by treating with 0.1% sodium lauryl sulfate or by heating to 60 °C for 10 min prior to assaying with S1 nuclease. When thus treated, duplex fragments neither form acid-soluble products nor show any change in molecular weight when measured by agarose gel electrophoresis (Kornberg, 1974). We infer that U-protein is required both to initiate unwinding and to maintain the partially unwound state; its removal or heat inactivation results in a snapback of the separated strands to restore the duplex condition.

Evidence that the limit to duplex unwinding is independent of time of incubation or of U-protein concentration is shown in Figure 6. Each of the samples of 400 and 800 bp duplexes attained maximal unwinding within 20 min and increases in protein/DNA ratios beyond 4–6 were without significant effect

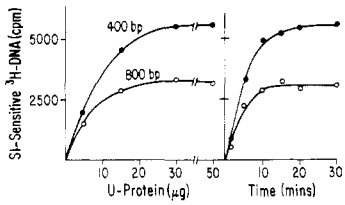

FIGURE 6: DNA unwinding and fragment size. Reactions were carried out as in Materials and Methods, section 6. Fragments were prepared as in Materials and Methods, section 5a. Each reaction mixture contained 5 μg of [^{3}H]DNA (26 000 cpm). For experiments in which the amount of U-protein was varied, the incubation time was 30 min. In the other experiments, 50 μg of protein was used per assay. Unwinding is expressed as a function of the radioactivity rendered acid soluble in the presence of S1 nuclease (Materials and Methods, section 6).

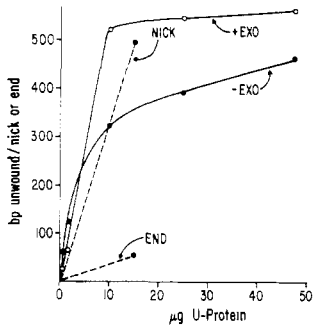

FIGURE 7: Unwinding of ΦX-174 DNA by lily U-protein. Incubations were carried out under standard conditions except for the volume of reaction mixture which was 50 μL. Two kinds of experiments are included in the figure. (1) The dashed lines compare unwinding at nicks and ends by using open circular DNA and linear ds-DNA as substrates. The linear DNA was prepared by treating RF II DNA with S1 nuclease. Half the products (by weight) had a molecular weight equivalent to RF II; the other half had an average molecular weight equivalent to ½RF II. Unwinding was calculated on the basis of 1.5 nicks or 3 ends per equivalent RF II (5375 bp). (2) The solid lines compare the kinetics of unwinding at nicks and gaps. Half of a RF II sample was treated with exonuclease III for 5 5 min at 35 °C (2 units of enzyme in 50 μL of solution containing 5 μg of DNA). The gaps thus formed had an average length of 100 bases per gap. The gapped DNA ("+ EXO") and the nicked DNA ("−EXO") were incubated in unwinding medium. The ordinate represents the number of bp per nick, gap, or end that is rendered acid soluble in the S1 nuclease assay.

on their respective levels of unwinding. We have determined that the limited unwinding is not due to a complete digestion of a small fraction of duplexes in the sample; agarose gel electrophoresis indicated a general reduction in molecular size following the S1 treatment (data not shown). Duplexes remaining after the unwinding of 100 bp fragments were used to demonstrate that residual duplexes are susceptible to additional unwinding by U-protein. The data in Table III indicate that the residues remaining from the 100-bp preparation were almost totally unwound on reincubating with U-protein.

Although a precise value for the number of bp unwound per duplex end cannot be obtained because of the spread in sizes within any one group of fragments, it is apparent from the results with nicked T7 DNA (Table III) that nicks are unwound to a greater degree than ends. Even if one assumes bidirectional unwinding, five times as much unwinding occurs in any one direction at a nick than at an end. This disparity in unwinding behavior was further studied by using ΦX-174 DNA as substrate. A preparation of RF II forms was divided into three groups; one was retained as such, a second was treated with S1 nuclease to produce linear duplexes, and a third was treated with exonuclease III to convert nicks into gaps. Each group was used as substrate for the U-protein. The results

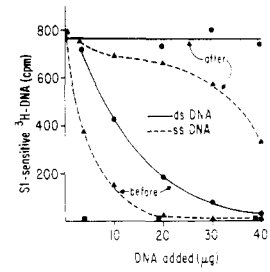

FIGURE 8: Stability of U-protein:duplex DNA complex; 0.2 μg of 800-bp [^{3}H]DNA fragments were assayed for unwinding with 20 μg of U-protein. Unlabeled ds- or ss-DNA was added either before or 10 min after the mixing of [^{3}H]DNA with U-protein. ATP was present at all times. The mixture of labeled DNA was incubated with U-protein for 15 min prior to S1 nuclease treatment. Total cpm in each assay was 4580. Squares represent values obtained in absence of U-protein.

of the analyses are shown in Figure 6. Using the same U-protein preparation, one comparison was made between the behavior of nicked circular (RF II) and the intact linear duplex DNA. In line with the data in Table III, ten times as many bp were unwound at the nicked site than were unwound at each of the ends. A second comparison was made between nicks and gaps in circular DNA. A time course for the unwinding of each of these circular forms is shown. It would appear that unwinding at gaps occurs more rapidly than at nicks, although the persistence of some exonuclease activity in the gapped DNA cannot be excluded. Even so, the difference in extent of unwinding between exonuclease treated and untreated duplexes is minor compared with that observed between nicks and ends. Moreover, in other experiments (data not shown) the interval of pretreatment with exonuclease was varied between 5 and 20 min using either 2 or 5 units of enzyme, but no major differences were observed between these various treatments with respect to the extent of duplex unwound by U-protein (Figure 7). We conclude that unwinding by the lily U-protein preparation is more extensive at nicks than at ends and that the presence of single strand gaps adjacent to duplex stretches has only a minor effect on the extent of unwinding. Whether the two types of unwinding belong to a single protein or to different proteins remains to be determined.

A high stability of the complexes formed between DNA and U-protein could at least partly explain the limited unwinding observed. To examine this possibility, 0.2 μg of [^{3}H]DNA was incubated with 20 μg of U-protein and the radioactivity released by S1 nuclease was measured in the presence of different amounts of unlabeled DNA which were added either before or after mixing the [^{3}H]DNA with U-protein. The results are summarized in Figure 8. As expected, addition of increasing amounts of unlabeled DNA (ss or ds) prior to U-protein dilutes the label correspondingly and decreases the amount of radioactivity released by S1 nuclease. ss-DNA clearly has a higher capacity or affinity for the protein than does ds-DNA. The initial decrease is less than expected because of the excess of U-protein relative to the [^{3}H]DNA. Addition of unlabeled DNA after the [^{3}H]DNA was reacted with U-protein demonstrates the stability of the complex. Even at a 200-fold dilution of the [^{3}H]DNA, unlabeled ds-DNA has no detectable effect on the amount of radioactivity released by S1 nuclease; there is thus very little interchange, if any, between bound and free ds-DNA fractions during 15 min of incubation. A 200-fold dilution with unlabeled ss-DNA, on the other hand, results in a 60% decrease in released radioactivity; however, no appreciable effect is observed at dilutions below 50. We infer the existence of a comparatively stable association between the ends of duplex DNA and U-protein. Whether a similar sta-

bility prevails at nicked sites remains to be determined. It was possible with the material available to make a preliminary test for a rapid exchange of U-protein between nicked [^{3}H]T7 DNA and nicked *E. coli* [^{14}C]DNA. The T7 DNA was preincubated with a limiting amount of U-protein under the same conditions as described above and *E. coli* DNA then added in ratios up to 3. No significant reduction in T7 unwinding could be detected nor could any significant unwinding of *E. coli* DNA be found. It appears unlikely that a rapid exchange of protein occurs at nicked DNA sites.

Unwinding and ATPase Activity. ATP hydrolysis by U-protein/DNA complexes exceeds the energetic requirements of duplex unwinding because such hydrolysis occurs not only in the absence of unwinding, but does so at a much faster rate in the presence of ss-DNA. To distinguish between the hydrolysis of ATP which is related to unwinding and hydrolysis related to the interaction between ss-DNA and protein, the ATPase activity was measured immediately following addition of ATP to the complex. The results are shown in Figure 9. If DNA is present in excess, ds-DNA initially promotes a higher level of ATPase activity than does ss-DNA. Within 1 min, however, the ds-DNA rate begins to drop and falls below that sustained by ss-DNA (Figure 9). With U-protein in excess, the curve of ATP hydrolysis in the presence of ds-DNA remains biphasic, but the ss-DNA supports a much higher rate of ATP hydrolysis presumably because of its much greater binding to the protein (Figure 9). To determine whether ds-DNA/protein complexes support a steady rate of ATP hydrolysis following their formation and partial unwinding, complexes were first formed in the presence of ATP, and then isolated, washed, and resuspended in fresh medium. Under these conditions, the slope of ATPase activity is not biphasic but is linear from the start as is the curve for ss-DNA (Figure 9). The data are consistent with the view that the protein catalyzes two different kinds of ATPase activities, one related to DNA unwinding and the other to an ss-DNA mediated ATPase activity.

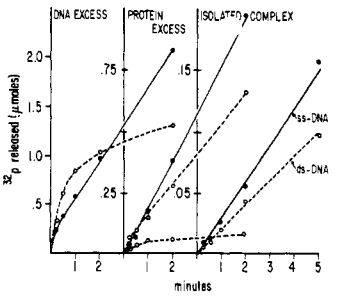

FIGURE 9: Kinetics of ATPase activity on mixing U-protein with DNA. Reactions were carried out in a volume of 0.35 mL under standard conditions for ATPase assay in the presence of [γ-^{32}P]ATP (8000 cpm/mmol). The "DNA Excess" mixtures contained 150 μg of ss- or ds-[^{3}H]DNA and 50 μg of protein. The "Protein Excess" mixtures contained 100 μg of protein and either 10 (lower dashed curve) or 100 μg of ds-DNA, or 10 μg of ss-DNA. "Isolated complex" was prepared from ^{3}H-labeled lily DNA which was sonicated to an average size of about 400 base pairs. Twenty micrograms of the duplex or 150 μg of the denatured fragments was incubated with 150 or 500 μg of protein, respectively, in the presence of ATP under standard conditions for DNA unwinding, and the mixture was then centrifuged in a 10–30% glycerol gradient. Centrifugation was carried out in a SW 41 rotor (Beckman) at 38 000 rpm for 24 h. The radioactive fractions from the lower half of the tube were pooled and concentrated by dialysis against 40% poly(ethylene glycol), followed by dialysis against 0.01 M Tris:dithiothreitol and then 0.1 mM Tris:dithiothreitol (pH 7.5). A portion of the DNA–protein complex thus isolated was incubated under standard conditions for ATPase assay. The ss-DNA isolated complex contained 2 μg of DNA; the ds-DNA complex contained 10 μg of DNA.

Discussion

A general outcome of this study is the demonstration that *Lilium,* a higher eukaryote, has one or more ATP-dependent unwinding proteins which are similar to some found in *E. coli.* In previous publications we described a protein from the meiotic cells of lilies and rats, which is very similar in its properties to the gene-32 protein (Hotta & Stern, 1974; Mather & Hotta, 1977). The similarity between prokaryotes and the meiotic tissues of eukaryotes with respect to proteins which unwind or reanneal DNA is impressive. Herrick & Alberts (1976) have isolated several DNA binding proteins from thymus tissue which also have similarities with the gene-32 protein, although less so than the meiotic proteins (Mather & Hotta, 1977). Our studies have been directed at meiotic tissue, primarily from the standpoint of identifying those proteins which are functional in meiotic recombination. That some differences have been found between the lily U-protein and corresponding microbial U-proteins is not surprising, given the differences in genome organization between the two groups of organisms. Whether it is genome organization in general or meiotic functions in particular that account for the differences observed cannot yet be determined. We have selected one from among four DNA-dependent ATPases for study and we are comparing it with two among probably several such proteins in *E. coli.*

There are three differences in property between the lily and microbial proteins which probably have important functional implications. (1) Bacterial U-proteins I and II unwind DNA/RNA hybrids whereas the lily U-protein has no such unwinding activity, at least with ΦX-174 hybrids as test substrates. (2) Bacterial U-proteins do not bind significantly to intact duplex DNA (Abdel-Monem et al., 1977a–c), whereas lily protein does bind at duplex ends. Moreover, a 3′-OH terminus in the DNA is required to effect binding with lily protein, but such a requirement has not been reported for the bacterial proteins. It is conceivable that binding in lily does not occur at ends but is due to nicks in the DNA or to some very short single-stranded tails at the ends of the duplexes, but, for reasons given under Results, we consider the possibility to be unlikely. (3) *E. coli* proteins I and II require single-stranded DNA adjacent to duplexes in order to effect unwinding. The lily protein appears to be capable of initiating unwinding at either the ends of a duplex or at nicks within the duplex. Converting nicks into gaps does not have a major effect on the unwinding capacity of the lily U-protein.

It is important to note that differences in methodology might account for some of the differences observed in unwinding characteristics. Although an S1 nuclease unwinding assay has been used for both organisms, procedures have been different. In the case of *E. coli,* S1 nuclease was applied after the DNA and U-protein mixtures were deproteinized. This was done to avoid the protective action of protein against S1 digestion (H. Hoffmann-Berling, personal communication). In the case of *Lilium,* S1 was applied without deproteinization under conditions which permitted the selective hydrolysis of ss-DNA. Had we followed the procedure used for *E. coli* proteins, we would not have detected unwinding activity except for very small duplex fragments. Our inability to detect unwinding follows from the fact that, in all cases studied, the extent of unwinding is limited to an apparently fixed number of bases. This property is in itself of considerable interest since it poses the question of how the unwinding process is consummated.

One would expect unwinding proteins of *E. coli* and *Lilium* to exercise comparable, though not necessarily identical, molecular functions. What needs to be considered is whether the

presumed or suggested functions of the unwinding proteins in *E. coli* throw any light on the specific role which the U-protein might play in meiotic cells of lily and whether the distinctive properties of the lily U-protein point in any particular direction with respect to function. The U-protein II of *E. coli*, or "rep" protein, has been assigned a role in ΦX-174 replication (Scott et al., 1977), but we doubt that a direct extrapolation of a replicative role from phage to lily meiocytes is warranted. We have already noted in the introductory section that the lily U-protein is most prominent during the interval of pairing and crossing-over and not during the interval of chromosome replication. To our knowledge, there are no other established functions of these bacterial U-proteins that would point to a specific role for the lily U-protein. Nor can any specific role be identified on the basis of the particular properties described, unless that role is defined in the general sense of unwinding being a required function for effecting recombination between DNA duplexes. There remains, of course, the still unexamined molecular issue of how the lily U-protein, which can unwind pure DNA in vitro, accomplishes unwinding in the context of nucleosome organization.

Acknowledgment

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Asymmetry of Chromatin Subunits Probed with Histone H1 in an H1–DNA Complex†

Kenshi Hayashi,* Thomas Hofstaetter,‡ and Naoko Yakuwa

ABSTRACT: Treatment of nucleosomes with a low concentration of sodium dodecyl sulfate removed all proteins except histone H1 from DNA, thus confirming our previous observation on sheared chromatin. No redistribution of H1 occurred during this procedure for isolation of the H1–DNA complex. The H1–DNA complex was isolated from a nucleosome monomer, doubly labeled in its protein and DNA and fractionated according to the length of DNA, and then the distribution of H1 was analyzed quantitatively. The results indicated that the monomer consisted of two subspecies, one containing 160 base pairs of DNA and one molecule of H1, and the other containing 140 base pairs of DNA and no H1. Since no monomer with two molecules of H1 was found, it is concluded that the nucleosome core has a binding site for H1 on only one side, and thus that the nucleosome is not a dyad.

Eukaryotic chromatins are all composed of a linear array of nucleosomes (υ bodies or chromatin subunits), like a "string of pearls" (Olins & Olins, 1974; Felsenfeld, 1975). These nucleosomes consist of an octamer of histone molecules (two molecules each of H2A, H2B, H3, and H4) and 140 base pairs of DNA.

Histone H1 is found in stoichiometric amount in chromatin and it is thought to be one of the building blocks of chromatin. H1 is believed to be located at the linker DNA, because the nucleosome core does not contain this histone (Varshavsky et al., 1976; Whitlock & Simpson 1976; Shaw et al., 1976; Noll

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