Monomer nucleosomes purified on isokinetic sucrose gradients are shown to dissociate into component DNA and histones at physiological ionic strength upon dilution to a DNA concentration below 20 μg/ml. The starting material is 11S, contains 145-190 bp DNA, and equimolar amounts of the four core histones with slightly less H1. Dilution of monomers in the presence of 0.14 M NaCl results in the rapid conversion of 10-40% of the 3H thymidine labeled material from 11S to 5S (5S is coincident with the S value of monomer length DNA). The proportion of nucleosomes which dissociate increases with increasing NaCl concentration between 0.15 M and 0.35 M and decreases with increasing DNA concentration above 1 μg/ml. Recycling 11S monomers, which remain after dissociation, through a second dilution in salt generates an equivalent proportion of 5S material as seen after the initial dilution. Thus, the dissociation does not result from special properties of a subset of nucleosomes. An equilibrium between intact monomer and free DNA and histones appears to be rapidly established under the conditions described and the dissociated DNA will reassociate with histones to form 11S monomers if conditions of high DNA concentration and low ionic strength are reestablished.

INTRODUCTION

The composition of nucleosomes, the basic repeating unit of eucaryotic chromatin, has been well documented and the interactions of the core histones, H2A, H2B, H3, and H4, with each other and with DNA have been extensively studied (for reviews, see 1,2,3). Many studies on histone-histone and histone/DNA interactions are, of necessity, performed in the presence of urea or in high or low ionic strength due to the tendency of chromatin and histones to aggregate at physiological salt concentrations. Since changes in nucleosome conformation and component interactions during DNA replication and RNA transcription are of primary biological interest, and since these processes occur at intermediate (i.e., physiological) ionic strength, it is difficult to relate interaction studies performed under nonphysiological conditions to changes which occur in nucleosome structure. Details of the interactions between histones and DNA at intermediate ionic strengths will facilitate our
understanding of the way in which these interactions may be altered during transcription and replication. The experiments reported in this paper provide data which indicate that a reproducible fraction of monomer nucleosomes, generated by micrococcal nuclease digestion of mouse tissue culture cell nuclei, is capable of rapid but apparently reversible dissociation into histones and DNA upon incubation at physiological ionic strength, pH, and temperature.

**EXPERIMENTAL PROCEDURES**

**Cell culture and labeling.** Mouse L929 cells (American Type Culture Collection) were grown in suspension culture in Joklik modified MEMS (Gibco) containing 10% fetal calf serum (Flow Labs). Logarithmically-growing cells were labeled with $[^{3}H]$ (methyl) thymidine (New England Nuclear) 2 $\mu$Ci/ml for 24 h, or $[^{14}C]$ lysine (Amer- sham) 25 $\mu$Ci/ml for 48 h.

**Preparation of nuclei and nucleosomes.** All manipulations were carried out on ice unless otherwise specified and all glassware was treated with siliclad. Five hundred ml of cells at $8 \times 10^5$ cells/ml were collected by centrifugation at 2000 rpm for 10 min, washed with Hanks balanced salt solution (Ca$^{++}$ and Mg$^{++}$ free), and nuclei were isolated according to Noll et al. (4) using homogenization and incubation buffers described by Hewish and Burgoyne (5). Washed cells were resuspended in homogenization buffer (10 mM Tris, 3 mM CaCl$_2$, 0.25 M sucrose, 1% TX100, pH 8.0), and broken by 10 strokes of a glass-teflon homogenizer. Nuclei were washed three times in incubation buffer (15 mM Tris, 0.25 M sucrose, 0.06 M KCl, 0.015 M NaCl, 0.015 mM spermine, 0.5 mM spermidine, 0.015 mM mercaptoethanol, pH 7.4), and resuspended in a final volume of 2.0 ml for digestion with micrococcal nuclease (Worthington). Nuclei were made 1 mM CaCl$_2$ and incubated at 37°C for 10 min prior to addition of enzyme. Micrococcal nuclease was added to 500 units/28 A$_{260}$ units of nuclei and digestion was allowed to proceed for 4-6 min at 37°C. Digestion was terminated by placing sample tubes on ice followed by the addition of 1/50 volume of 100 mM EDTA. Digested nuclei were sedimented at 3000 rpm in a Beckman Ja-20 rotor for 15 min. After centrifugation, the supernatant was removed and the nuclei were swelled in 0.2 mM EDTA, pH 8.0, for 10 min on ice before being lysed by several passages through a 27 gauge needle. The solution of nuclei was centrifuged at 6000 rpm in a Beckman J-13 rotor for 15 min and the supernatant was layered on a 5-28.8% isokinetic sucrose gradient containing 1 mM EDTA, pH 7.0, with $C_t =$
Samples were centrifuged for 24 h at 26,000 rpm in a Beckman SW27 rotor. Gradients were collected by pumping from the bottom of the tube and absorbance at 260 nm of each fraction was determined. Fractions comprising the center of the monomer peak were pooled, dialyzed overnight against 10 mM Tris, 1 mM EDTA, pH 7.4, and stored at 5°C. DNA concentrations were estimated assuming 1 A260 unit = 22 μg/ml DNA in chromatin (6). All monomer preparations had an A260/A280 ratio of ~1.6.

Incubation and centrifugation conditions.

Sucrose gradient purified monomer nucleosomes were diluted to the desired final DNA concentration in the appropriate buffer at 5°C or 37°C for various periods of time as specified in the Results. Components of the incubation mixture were mixed thoroughly prior to the addition of monomers. After incubation, samples of these mixtures were layered on 5-27% isokinetic sucrose gradients containing 1 mM EDTA (C5% = 5%, C27% = 27%, Vm = 9.45 ml) and centrifuged in a Beckman SW41 rotor at 32,000 rpm for 15 h. Approximate S values were determined as described by McCarty et al. (7). Gradients were fractionated into siliconized tubes by pumping from the bottom of the tube and samples were counted in Aquasol-2 (New England Nuclear).

Carrier experiments.

Nucleosome monomers were prepared as described above from unlabeled cells. The cold monomers were added as a carrier to rapidly increase the final DNA concentration in specified experiments. Concentrations were determined as stated above. Addition of carrier monomers was followed by dialysis of labeled sample plus carrier into the appropriate buffer using Spectropor 1 dialysis membrane (M.W. cutoff 6,000-8,000) (Scientific Products).

RESULTS

Micrococcal nuclease digestion products of 3H-TdR labeled mouse L-929 cells were separated on isokinetic sucrose gradients as described in Experimental Procedures and peak monomer fractions were pooled, dialysed overnight against 10 mM Tris, 1 mM EDTA (pH 7.3) and stored at 5°C. Proteins from each group of pooled fractions were run on 15% SDS acrylamide gels and these gels demonstrated the presence of the core histones in approximately equimolar amounts with slightly lower amounts of H1 (data not shown). Samples which were stored at 5°C for up to four weeks showed no obvious histone degradation as assayed by SDS gels. When samples of monomers which had been stored at 20-30 μg/ml DNA were rerun on isokinetic sucrose gradients containing 1 mM EDTA,
pH 7.0, they sedimented at 11S (Figure 1A). It was observed, however, that if the monomers were diluted to 2 μg/ml DNA in 0.15 M NaCl, 1 mM EDTA, 10 mM Tris, and incubated at 37°C for 2 h, a new peak was generated with a sedimentation coefficient of 5-6S (Figure 1B). The material in this peak represented from 20-50% of the input material in different experiments. That is, different preparations of monomers varied in the amount of 5S material seen upon dilution into the buffer described above, but the amount of 5S material was constant within a single preparation under any given set of conditions of ionic strength, DNA concentration, and temperature. The appearance of material sedimenting at 5S was not inhibited by the addition to the mixture of 1 mM PMSF or 50 mM sodium bisulfite as protease inhibitors, and the amount of 5S material was not reduced by increasing the EDTA concentration (data not shown). These experiments indicate that the appearance of

Figure 1. (A) Monomer fractions stored at 20 μg/ml in 10 mM Tris, 1 mM EDTA, resedimented as described in Experimental Procedures. (B) Monomers, diluted to 2 μg/ml in 0.15 M NaCl, 10 mM Tris, 1 mM EDTA, and incubated at 37°C for 2 h prior to loading samples onto gradient. Sedimentation coefficients were calculated as described in Experimental Procedures.
material sedimenting at 5S is not a result of proteolytic or nucleolytic activity in the samples. In addition, comparable amounts of 5S material are produced at DNA concentrations from 1-2 µg/ml when 250 µg/ml of normal rabbit immunoglobulin is included in the incubation mixture (data not shown). This argues that the release of 5S DNA does not result from adsorption of monomers to the glassware and subsequent release of DNA.

In order to understand the source of the 5S material, its composition was determined, and the conditions which would generate this material were characterized. Experiments were undertaken to determine both the size of the DNA component in the 5S peak and the presence or absence of histones in these fractions. Since very small amounts of material were used in these experiments, and the final concentration of protein and DNA were 1 or 2 µg/ml, both these questions were approached indirectly. To determine the presence and amount of histone associated with the 5S peak material, nucleosome monomers were isolated from cells labeled for 48 h with [14C] lysine. When these monomers were diluted to 2 µg/ml DNA in 0.15 or 0.25 M NaCl, 10 mM Tris, 1 mM EDTA, and sedimented as described previously, no counts above background were found in fractions corresponding to 5S (Figure 2). However, the number of counts migrating at US was reduced to 49% (in 0.15 M NaCl) and 42% (in 0.25 M NaCl) of the input counts. No 14C was found at the top of the gradient. Since the maximum amount of histone lost from the US peak in this experiment was estimated to be only 0.5 µg, we reasoned that such a small amount of protein might adsorb to the walls of the centrifuge tube during gradient fractionation. Thus each tube was rinsed with 0.5% SDS; 60-100% of the 14C counts which were lost from the monomer peak was found in the SDS rinse. These data suggest that under the incubation conditions described above, a portion of monomer nucleosomes dissociates into histone and DNA. To further substantiate this hypothesis, DNA was extracted from monomers, sized on 5% acrylamide gels, and sedimented in a 5-27% isokinetic sucrose gradient as detailed in Experimental Procedures. The purified DNA ranged from 145-190 BP in length and migrated at 5S (Figure 3). From these two types of experiments, we concluded that the 5S material generated at the expense of 11S monomers is monomer size histone-free DNA. Nucleosome dimers will also undergo dissociation upon dilution of the dimers to 2 µg/ml DNA in the buffer described above, resulting in broad peaks sedimenting slightly faster than 11 and 5S (data not shown).

Extreme conditions, such as low pH, high salt (e.g., > 2 M), or SDS, are usually required to separate histones and DNA. Because the dissociation shown here occurs at physiological salt and pH, we further characterized the
Figure 2. Profiles of $[^{14}C]$ lysine labeled nucleosome monomers. (Δ) Monomers diluted to 10 μg/ml in 10 mM Tris, 1 mM EDTA. (●) Monomers diluted to 2 μg/ml in 0.15 M NaCl, 10 mM Tris, 1 mM EDTA. (○) Monomers diluted to 2 μg/ml in 0.25 M NaCl, 10 mM Tris, 1 mM EDTA. Samples were loaded directly onto the gradient following dilution into the appropriate buffer.

Figure 3. 1 μg of extracted DNA sedimented as described in Experimental Procedures. Sedimentation coefficient calculated as described in Experimental Procedures.
effect of DNA concentration, ionic strength, and time of incubation on the extent of dissociation.

To determine the effect of DNA concentration appropriate amounts of nucleosome monomers were diluted to different final concentrations of DNA in 0.15 M NaCl, 10 mM Tris, 1 mM EDTA, pH 7.3, incubated at 37°C for 2 hr and centrifuged as described in Experimental Procedures. As shown in Table 1, 5S material was generated at concentrations below 20 mg/ml DNA. For DNA concentrations of 10 and 5 μg/ml, 22% and 23.4% of input counts were converted to 5S with the remainder of the counts at 11S. At a DNA concentration of 1 μg/ml, about 40% conversion was observed. Thus, the dissociation of monomers is DNA concentration dependent but does not display a simple linear relationship. If we assume a simple reversibly dissociating system, then the following expression should hold: (c-x) → x + x where c = the initial concentration of monomer; x = the concentration of the dissociated species; x/c = the fraction dissociated; and k = the dissociation constant. For a given x/c one can calculate the k = x^2 / (c-x). When this was done using the experimental values of x/c given in Table 1, the k's are reasonably close, with the exception of that calculated for the lowest DNA concentration (see Table 2). This discrepancy could arise from the difficulty in accurately measuring this amount of material. For k = 0.1 we calculated the predicted x/c for the initial DNA concentrations listed in Table 1. These calculated values appear in the last column of Table 2 and are somewhat lower than those measured experimentally. However, since values for k are based on the experimental determinations of x/c from sucrose gradients, this difference is probably with-

<table>
<thead>
<tr>
<th>DNA Concentration</th>
<th>Percent Counts at 11S</th>
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<tbody>
<tr>
<td>20 μg/ml</td>
<td>100 %</td>
</tr>
<tr>
<td>10 μg/ml</td>
<td>78 %</td>
</tr>
<tr>
<td>5 μg/ml</td>
<td>76.6%</td>
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<tr>
<td>2 μg/ml</td>
<td>72.6%</td>
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<tr>
<td>1 μg/ml</td>
<td>60 %</td>
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Table 1. Effect of DNA concentration on the extent of nucleosome dissociation. Monomers were diluted to the DNA concentration indicated in the table in 10 mM Tris, 1 mM EDTA, 0.15 M NaCl, pH 7.3, incubated at 37°C for 2 h and sedimented as described in Experimental Procedures. The percentage of counts in the 11S peak at the various DNA concentrations was calculated.
Table 2. Values for $k$ are calculated as described in text using the experimentally determined values for the fraction dissociated based on figures in Table 1. Expected values for $\frac{4S}{5S}$ were calculated using $k = 0.1$.

In the limits of accuracy of the experimental design. At higher DNA concentrations where no clear 5S peak was visible, it remains possible that there is dissociated DNA but that it is below the resolution of the experiment. This is suggested by the appearance of a shoulder which extends from the light side of 11S into the area where 5S DNA would be found in heavily loaded gradients using samples with DNA concentrations of $\geq 20 \mu g/ml$.

The effect of the final concentration of NaCl on the extent of dissociation was examined by diluting five samples of monomers to $1 \mu g/ml$ DNA in $10 \text{mM Tris, } 1 \text{mM EDTA}$ with final NaCl concentrations at 0, 0.05 M, 0.15 M, 0.25 M, and 0.35 M. Table 3 details the results of these experiments. Little or no dissociation occurs below 0.05 M NaCl and the maximum amounts of dissociation we have observed occurs at 0.25 M NaCl. Thus, the dissociation of the

<table>
<thead>
<tr>
<th>(NaCl) in 10 mM Tris, 1 mM EDTA</th>
<th>Percent Counts at 11S</th>
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<tbody>
<tr>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>0.05 M</td>
<td>71%</td>
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<tr>
<td>0.15 M</td>
<td>62%</td>
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<tr>
<td>0.25 M</td>
<td>44%</td>
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<tr>
<td>0.35 M</td>
<td>44%</td>
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Table 3. Effect of NaCl concentration on the extent of nucleosome dissociation. Monomers were diluted to $1 \mu g/ml$ DNA in $10 \text{mM Tris, } 1 \text{mM EDTA}$, containing 0-0.35 M NaCl as indicated in the table. Sedimentation was done as described in Experimental Procedures and the percentage of counts found in the 11S peak was calculated.
major components of the monomer particle is a combined effect of dilution and increasing the ionic strength to at least 0.1 M NaCl. Little or no dissociation is found when only one of these conditions is met.

We have also examined the effects of incubation time and temperature at the level of resolution which our experimental procedure allows. Incubation of monomers at 2 µg/ml DNA in 0.15 M NaCl, 10 mM Tris, 1 mM EDTA for 1, 2, 3, and 4 h showed some variation, but neither a consistent increase or decrease in the percent of material sedimenting at 5S (Table 4). In addition, samples diluted to 2 µg/ml DNA in 0.25 M NaCl, 10 mM Tris, 1 mM EDTA and sedimented immediately and 24 h later showed no change in the amount of dissociation. Also, samples which were diluted into buffer at either 5° or 37°C and loaded directly on the gradient showed the same amount of dissociation as those incubated 1-4 h (Table 4). Thus, dissociation is rapid in that it occurs within the time it takes to mix the sample and load it on the gradient; and, within our ability to measure it, is temperature independent between 5° and 37°C.

Two additional approaches were taken to further characterize the nature of the dissociation phenomenon. In order to determine whether or not dissociation is reversible, 11S monomers at 24 µg/ml DNA were diluted to 2 µg/ml in 0.25 M NaCl, 10 mM Tris, 1 mM EDTA, resulting in ~40% dissociation. A portion of this sample was made 18 µg/ml DNA by the addition of cold carrier and dialyzed against 10 mM Tris, 1 mM EDTA to remove NaCl. The results of such an experiment are illustrated in Figure 4. The disappearance of material at 5S and shift in labeled DNA to the 11S position indicates that the

<table>
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<th>Time of incubation at 37°C before loading onto gradient</th>
<th>Percent of counts remaining 11S</th>
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<tbody>
<tr>
<td>0 (5°)</td>
<td>73%</td>
</tr>
<tr>
<td>1 h</td>
<td>73%</td>
</tr>
<tr>
<td>2 h</td>
<td>80%</td>
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<tr>
<td>3 h</td>
<td>84%</td>
</tr>
<tr>
<td>4 h</td>
<td>68%</td>
</tr>
</tbody>
</table>

Table 4. Effect of time and temperature on the extent of nucleosome dissociation. Monomers, diluted to 2 µg/ml in 10 mM Tris, 1 mM EDTA, 0.15 M NaCl, were incubated for the times listed in the table at 5°C or 37°C as indicated prior to sedimentation as described in Experimental Procedures. The percentage of counts found at 11S was calculated.
dissociation observed at low DNA concentration in 0.25 M NaCl is reversible. Although we cannot rigorously exclude the possibility that the DNA reassociates with excess octamers in the carrier monomer preparation as described by Stein (9), we have observed conversion of 35% of the 5S DNA to 11S by dialysis and concentration from 2 to 7 µg/ml in the absence of carrier (data not shown). This decrease in amount of 5S DNA upon concentration is consistent with the expected difference in amounts of 5S DNA found at final DNA concentrations of 2 and 7 µg/ml. These data suggest that the dissociated DNA and histone can reassociate to form an 11S particle in the absence of carrier monomers.

Experiments were also performed to determine if the dissociation of 11S monomers into DNA and histones is an equilibrium phenomenon or if it reflects two kinds of nucleosomes in the preparation which differ by their susceptibility to dissociation at low DNA concentrations in physiological salt. Monomers at 2 µg/ml DNA in 0.25 M NaCl, 10 mM Tris, 1 mM EDTA, pH 7.3, were sedimented as described in Experimental Procedures and the peak 11S fractions were pooled. Carrier monomers were added to the pooled 11S fractions and the mixture was dialyzed against 10 mM Tris, 1 mM EDTA, pH 7.3, to remove sucrose. The final concentration of labeled sample plus carrier was 11 µg/ml DNA and control experiments showed 100% of these monomers sediment at 11S. However, if the mixture is recycled through a second dilution to 2 µg/ml DNA and addition of NaCl to a final concentration of 0.25 M, the labeled monomers again dissociate to yield 72% of input counts at 11S and 27% at 5S, as shown in Figure 5. Thus, it seems that a rapidly established equilibrium exists be-
Figure 5. (A) Example of a gradient in which the three peak 11S fractions were pooled prior to addition of carrier and dialysis. Amount of dissociation in five similar gradients ranged from 20-28%. (B) Pooled monomer fractions to which carrier nucleosomes were added followed by dialysis against 10 mM Tris, 1 mM EDTA. Final DNA concentration in sample is 11 µg/ml. (C) Portion of sample described in (B), diluted to 2 µg/ml DNA in 0.15 M NaCl, 10 mM Tris, 1 mM EDTA and resedimented. Sample gave 28% dissociation.

tween intact and dissociated monomers.

DISCUSSION

We have shown that nucleosome monomers will undergo reversible dissociation into their component DNA and histones as a function of DNA concentration and ionic strength. Two trivial explanations for this phenomenon can be eliminated by our work. The possibility that 5S DNA was adventitiously bound to the original monomer preparation, and dissociated upon dilution and raising the ionic strength, is unlikely since monomer samples raised to the same ionic strength, but at a DNA concentration of 30 µg/ml, showed no evidence of 5S material (data not shown). Since increasing the concentration of EDTA did not reduce the amount of 5S material, although it would be expected to inhibit nuclease activity, we can also eliminate self-redigestion of chromatin as reported by Carter and Levinger (7) as an explanation for the appearance of 5S material under experimental conditions.

Within the resolution of our experiments, we find that the dissociation of monomers into DNA and histones occurs very rapidly, and when the time of incubation of monomers at low DNA concentration in 0.15 or 0.25 M NaCl is
extended to 24 h, there is no further dissociation. Simplistically, two possible explanations can account for these data. If we assume that all monomers can dissociate, but that dissociation is reversible, an equilibrium should be established after which it would appear that a constant amount of 5S DNA is being generated. Alternatively, there might exist a subset of nucleosomes which are capable of dissociating, possibly due to compositional or structural differences. Our data support the existence of an equilibrium between 11S monomers and the dissociated components and show that the dissociation does not result from a subset of nucleosomes which are selectively labile under our experimental conditions.

Further evidence for an association-dissociation equilibrium between histones and DNA derives from work done on reconstitution of monomers which are dissociated in 2.5 M NaCl and rapidly diluted to 0.6 M NaCl (8). These data show that initially only 80% of the DNA is rapidly reconstituted into monomer while 20% remain dissociated. If the rapidly reassociating fraction of monomers is recycled through the salt-jump procedure, again only 80% of the starting material reassociates rapidly indicating that there is not a specific subpopulation which differs in its ability to reassociate.

Two other published studies are relevant to the work described here. Stacks and Schumacher (9) described the dissociation of nucleosomes at salt concentrations between 0.25-1.0 M NaCl at DNA concentrations between 50 and several hundred µg/ml. They observed a salt and DNA concentration dependent dissociation of particles with kinetics which suggested a slow reversibly-dissociating system. Although the trend of increased dissociation with increased salt and decreased DNA concentration is analogous to the data presented here, the difference in the dissociation rates and the fact that these workers used substantially higher concentrations of both DNA and salt make it impossible to directly relate the two dissociation phenomena. Since the NaCl concentrations used in the studies by Stacks and Schumacher are sufficiently high to induce both histone mobility and partial histone dissociation while our studies are at salt concentrations below these levels, it is possible that the basis for dissociation is quite different.

Another study by Lilley et al. (10) described nucleosome dissociation under conditions very similar to those we have discussed. In studies in vitro transcription of monomer nucleosomes by RNA polymerase II, these workers describe the generation of free DNA when monomers at 50 µg/ml DNA or less were incubated for 20 minutes at 25°C in the presence of increasing concentrations of (NH₄)₂SO₄. At 150 mM salt, nearly complete dissociation was observ-
ed. In addition, monomers stored for increasing periods of time prior to assay exhibited increasing amounts of dissociation under a given set of conditions. In contrast to this finding, we have seen no substantial increase in dissociation of monomers upon storage.

The work of Lilley and coworkers emphasizes the situations in which this nucleosome dissociation may become a problem. Their observations were made during attempts to set up an in vitro transcription system with monomer nucleosomes. In order to be in enzyme excess, the reactions were run at about 1 µg/ml of DNA, a concentration at which nucleosome monomers dissociate as shown by their data and that presented above. Similar dissociation would be expected during reconstitution experiments using cloned DNA fragments since quantities sufficient to allow experiments to be performed at DNA concentrations greater than 20 µg/ml may not be available. Thus, the dissociation phenomenon detailed here is relevant to the stability of monomer nucleosomes under conditions which may be utilized in a variety of in vitro reactions.

ACKNOWLEDGMENTS

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