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Genetics

Nucleosomes on linear duplex DNA allow homologous pairing but prevent strand exchange promoted by RecA protein

(nucleoprotein filaments/chromatin/gene targeting/genetic recombination)

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ABSTRACT **To understand the molecular basis of gene targeting, we have studied interactions of nucleoprotein filaments comprised of single-stranded DNA and RecA protein with chromatin templates reconstituted from linear duplex DNA and histones. We observed that for the chromatin templates with histone/DNA mass ratios of 0.8 and 1.6, the efficiency of homologous pairing was indistinguishable from that of naked duplex DNA but strand exchange was repressed. In contrast, the chromatin templates with a histone/DNA mass ratio of 9.0 supported neither homologous pairing nor strand exchange. The addition of histone H1, in stoichiometric amounts, to chromatin templates quells homologous pairing. The pairing of chromatin templates with nucleoprotein filaments of RecA protein–single-stranded DNA proceeded without the production of detectable networks of DNA, suggesting that coaggregates are unlikely to be the intermediates in homologous pairing. The application of these observations to strategies for gene targeting and their implications for models of genetic recombination are discussed.**

In recent years several laboratories have shown homologous recombination between DNA newly introduced into recipient mammalian cells and a target chromosomal gene. Such "gene targeting" has implications for the study of gene expression, the development of animal models for human genetic diseases, the improvement of livestock animals and plants, the production of products of pharmaceutical importance, and the repair of genetic defects (1, 2). Mouse embryonic stem cells have been used for gene targeting to create mutant genes and to correct mutant phenotypes (3–5). Thus, mammalian somatic cells do contain the enzymic machinery for homologous recombination (6–9). Capecchi (10) has shown that homologous recombination is maximal in S phase of the cell cycle and is manifested within 30 min after the DNA is injected into the nucleus.

The frequency of the occurrence of recombinants has been low and variable, depending upon the cell type and the locus selected (2). A robust targeting system has not yet emerged. Efforts to develop such a system should examine the DNA at the locus of interest when it is in an active state and when it is in a repressed state. Indeed, it has been illustrated that the state of chromatin determines the accessibility of DNA to various enzymatic processes (11). A transcriptionally active gene shows greater sensitivity to nucleases than does the repressed gene. This repression in eukaryotes is believed to be exerted by histones, the ubiquitous general repressors (12).

To investigate the molecular mechanisms of gene targeting, we chose the *Escherichia coli* RecA protein as a model since the system is well defined at the genetic and molecular levels (13, 14). With this system we have addressed two related issues: (*i*) what are the parameters of eukaryotic chromatin that determine the efficiency of gene targeting and (*ii*) how does chromatin structure influence homologous pairing and strand exchange of duplex DNA?

EXPERIMENTAL PROCEDURES

Enzymes, Proteins, and DNA. *E. coli* RecA protein was prepared by the method of Griffith and Shores (15) and its concentration was determined as described (16). *E. coli* single-stranded DNA binding protein (SSB) was prepared as described (17). Nuclei were prepared from adult rat liver as described by Rao *et al.* (18). Histone H1 was extracted with salt and core histones were extracted with acid (19). The Coomassie blue-stained SDS/polyacrylamide gels revealed that the histone preparation was neither degraded nor significantly contaminated with additional proteins. The histone preparations were devoid of exo- and endonuclease activities. Form I [^{3}H]DNA (negatively superhelical duplex DNA) from M13 and M13 Goril and unlabeled circular single-stranded DNA (ssDNA) were prepared as described (20). The concentration of DNA is expressed as moles of nucleotides.

Reconstitution of Nucleosomes. Reconstitution was accomplished as described (21), with the following modifications. Core histones were mixed with linear duplex [^{3}H]DNA at specified mass ratios in a buffer containing 10 mM Tris·HCl (pH 7.5), 0.2 mM EDTA, and 0.8 M NaCl in a total volume of 0.4 ml. The reaction mixture of histones and DNA was incubated at 37°C for 10 min, and the reconstitution was allowed to proceed at 4°C for 15–16 hr. The salt was dialyzed gradually (0.8 M → 0.4 M → 0.02 M NaCl) at 4°C using Centricon-10 microconcentrators (Amicon).

Analysis of the Reconstituted Chromatin Templates. Individual nucleosome preparations were subjected to *Hae* III, DNase I, and micrococcal nuclease digestion. Chromatin templates and naked duplex DNA (2 nmol) were digested with 16 units of *Hae* III in 70 μl of a standard assay buffer for 5 min at 37°C as described by the supplier (New England Biolabs). The digestion was terminated by adding SDS to 0.1%, EDTA to 25 mM, and proteinase K to 0.2 mg/ml and incubating at 37°C for 15 min. The DNA was electrophoresed on a 1.5% agarose gel using 89 mM Tris/89 mM borate/2 mM EDTA, pH 8.3 (Tris borate/EDTA).

Micrococcal nuclease digestion of chromatin templates at a histone/DNA (^{32}P labeled) mass ratio of 1.6 was done in a buffer containing 10 mM Tris·HCl (pH 7.5), 20 mM NaCl, 2 mM $CaCl_2$, 0.2 mM EDTA, and micrococcal nuclease (1 unit/ml) in a total volume of 50 μl. The reaction was done as described above. Each sample was loaded on a 7% polyacrylamide gel and electrophoresed at 30 V for 16 hr in Tris borate/EDTA.

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Abbreviations: SSB, single-stranded DNA binding protein; ssDNA, circular single-stranded DNA.

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Nitrocellulose Filter Assay for Joint Molecules. The assay was done as described (22). The preparation of nucleoprotein filaments and the formation of joint molecules was done in a standard assay buffer containing an ATP-regeneration system (23). The formation of joint molecules was initiated by the addition of either naked duplex DNA or reconstituted chromatin templates. Histone H1 was added to the reaction mixture, at least 1 min before to the addition of chromatin templates to initiate the formation of joint molecules.

Assay for Strand Exchange. Strand exchange was measured in an ATP-regeneration system (24). The reaction mixture contained 16 μM ssDNA, 8 μM RecA protein, 1 μM SSB, and 16 μM M13 linear duplex DNA or chromatin templates with a core histone/DNA mass ratio of 0.8. Samples were deproteinized in a reaction mixture containing 0.1% SDS, 25 mM EDTA, and proteinase K (0.2 mg/ml) for 15 min at 37°C. After the addition of tracking dye, individual samples were loaded on a 0.8% agarose gel and electrophoresed in Tris borate/EDTA at 40 V for 16 hr. The gel was stained with ethidium bromide at 0.5 μg/ml, destained, and photographed.

Coaggregation Assay. The assay is based on the method of Tsang *et al.* (25). The reaction mixture (30 μl) in a standard assay buffer containing 8 μM M13 ssDNA, 12 mM $MgCl_2$, 4 μM RecA protein, and an ATP-regeneration system was preincubated for 20 min at 37°C. M13 linear duplex [^{3}H]DNA or reconstituted chromatin template was then added. The samples were removed from the water bath, at the indicated time intervals, and immediately centrifuged in an Eppendorf microcentrifuge for 25 sec at 25°C. Radioactivity was measured as described by Tsang *et al.* (25).

RESULTS

Rationale and Experimental Design. The packaging of DNA into chromatin structures is believed to repress various processes involving DNA. Since the bulk of eukaryotic chromatin is not naked but is associated with histones in an array of nucleosomes, the question arises as to how homologous recognition, pairing, and strand exchange overcome this impediment. To investigate this we utilized reconstituted chromatin templates and nucleoprotein filaments of RecA protein–ssDNA as model system. The choice of a recombinase from *E. coli*, histones from rat liver, and DNA from phages may be intriguing but presents no conceptual problems. We have used linear duplex DNA and ssDNA as substrates since the assays are rapid and, as a consequence, have been used intensively in studies on the strand transferase activities in the prokaryotic as well as in the eukaryotic systems (7–9, 14, 26–30).

Construction and Characterization of Chromatin Templates. In the experiments described here, we used negatively superhelical [^{3}H]DNA (form I), isolated from phages M13 and M13 Goril to obtain the appropriate linear substrates (Fig. 1). The chromatin templates were assembled by mixing linear duplex [^{3}H]DNA with core histones at appropriate concentrations. We employed three independent criteria to assess the faithful reconstitution and integrity of chromatin templates.

The chromatin templates were digested with *Hae* III. As shown in Fig. 2*A*, the naked duplex DNA was completely digested whereas the chromatin templates formed at a core histone/DNA mass ratio of 0.8 were protected from digestion. Since digestion with *Hae* III indicates the accessibility of a small region on the DNA, we used DNase I to obtain a more generalized picture of nucleosome protection and to facilitate quantitative analysis. Whereas >90% of naked duplex [^{3}H]DNA became acid-soluble after 2 min of incubation with DNase I at 5 μg/ml, with chromatin templates and the same amount of DNAse I, the level of protection was

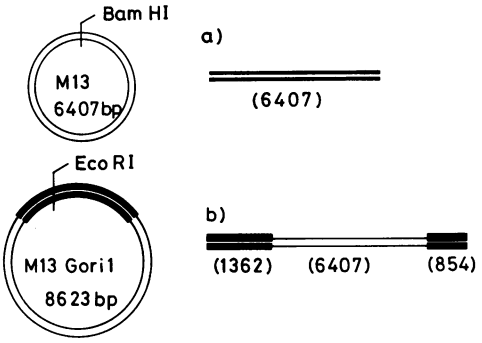

FIG. 1. DNA substrates. Thin and thick lines represent sequences derived from bacteriophages M13 and G4, respectively. Numbers in parenthesis indicate the length in base pairs (bp) of that region. Substrate a was prepared by cleaving M13 form I DNA with *Bam*HI and substrate b was prepared by digesting M13 Goril form I DNA with *Eco*RI.

dependent on the mass ratio of core histone/DNA; at 0.8, 1.6, and 9.0, the protection was 60%, 72%, and 92%, respectively (data not shown). Direct evidence for the nucleosomal structure of chromatin templates was deduced from micrococcal nuclease digestion. As shown in Fig. 2*B*, digestion of chromatin templates formed at a core histone/DNA mass ratio of 1.6 generated a nucleosome-resistant fragment of ≈180 base pairs. Increasing the time of digestion with micrococcal nuclease converted most of the high molecular weight DNA into pieces of 180 base pairs (Fig. 2*B*, lane e). To ascertain the efficiency of reconstitution, the assembled chromatin tem-

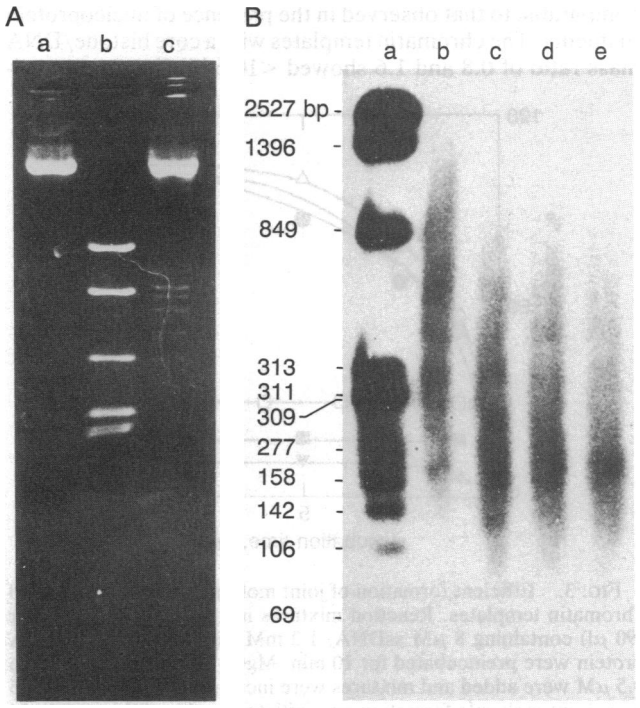

FIG. 2. Characterization of reconstituted chromatin templates. (*A*) Restriction endonuclease *Hae* III digestion. Naked duplex DNA and chromatin templates were digested with *Hae* III. Lanes: a, naked M13 linear duplex DNA; b, as in lane a but digested with *Hae* III; c, chromatin templates digested by *Hae* III. (*B*) Micrococcal nuclease digestion of chromatin templates. Reconstituted chromatin templates containing a core histone/DNA mass ratio of 1.6 were digested with micrococcal nuclease. Lanes: a, *Hae* III digest of ^{32}P-labeled M13 form I DNA; b, chromatin templates digested for 30 sec with micrococcal nuclease; c, same as lane b but digested for 1 min; d, same as lane b, but digested for 2 min; e, same as lane b but digested for 3 min.

plates were purified by neutral sucrose gradient centrifugation and were clearly separated from the unreconstituted DNA (data not shown).

Efficient Formation of Joint Molecules with Chromatin Templates. For the formation of plectonemic joint molecules requiring a free end in the region of homology (31, 32), we used chromatin templates formed with M13 linear duplex [^{3}H]DNA (Fig. 1, substrate a), and nucleoprotein filaments of RecA protein–M13 ssDNA. The pairing reaction, at least through the homologous alignment step, is governed by the conformation of ssDNA upon which RecA protein polymerizes (22). To delimit this step, prior to the addition of duplex substrates, we preincubated ssDNA with RecA protein and SSB to form stable and active nucleoprotein filaments of RecA protein–M13 ssDNA (23).

We started the pairing reaction by adding either naked duplex DNA or chromatin templates to nucleoprotein filaments of RecA protein–M13 ssDNA. As shown in Fig. 3, the formation of joint molecules with naked duplex [^{3}H]DNA reached a plateau by 5 min with a yield of >80%. Interestingly, the rate and extent of formation of joint molecules with chromatin templates containing a core histone/DNA mass ratio of 0.8 and 1.6 was indistinguishable from that with naked duplex DNA. In contrast, the yield of apparent joint molecules from chromatin templates with a histone/DNA mass ratio of 9 was measurable but remained constant throughout the incubation period. Insight into this phenomenon was derived from a control experiment. In one set of experiments, we tested the binding of chromatin templates to nitrocellulose filters in the absence of nucleoprotein filaments of RecA protein–M13 ssDNA. The extent of binding of chromatin templates with core histone/DNA mass ratio of 9.0 was comparable to that observed in the presence of nucleoprotein filaments. The chromatin templates with a core histone/DNA mass ratio of 0.8 and 1.6 showed <10% binding to nitrocellulose filters in the absence of nucleoprotein filaments of RecA protein–M13 ssDNA. We conclude that the apparent signal observed with chromatin templates with core histone/DNA mass ratio of 9.0 in the presence of nucleoprotein filaments is nonspecific binding to nitrocellulose filters.

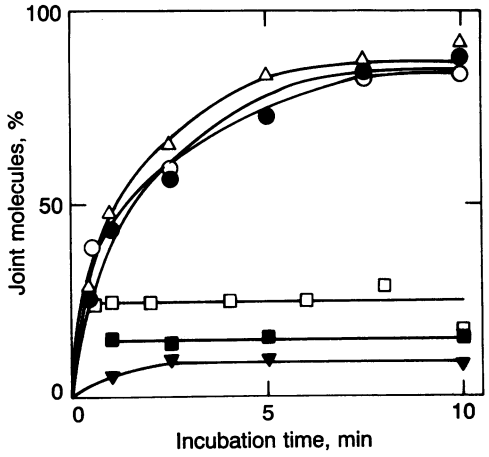

FIG. 3. Efficient formation of joint molecules with reconstituted chromatin templates. Reaction mixtures in a standard assay buffer (90 μl) containing 8 μM ssDNA, 1.2 mM $MgCl_2$, and 4 μM RecA protein were preincubated for 10 min. $MgCl_2$ to 12 mM and SSB to 0.5 μM were added and mixtures were incubated for an additional 5 min. Joint molecule formation was initiated by the addition of 5 μM naked linear duplex [^{3}H]DNA or reconstituted chromatin templates. Samples of 10 μl were removed and delivered into 5 ml of ice-cold 1.5 M NaCl/0.15 M sodium citrate and immediately filtered through a nitrocellulose filter. ○, Naked linear duplex [^{3}H]DNA; ●, reconstituted chromatin templates with a core histone/[^{3}H]DNA mass ratio of 0.8; △, reconstituted chromatin templates with a core histone/[^{3}H]DNA mass ratio of 1.6; □, reconstituted chromatin templates with a core histone/[^{3}H]DNA mass ratio of 9.0. Control reactions were done as above in the absence of nucleoprotein filaments of RecA protein–ssDNA. ■, Chromatin templates with a core histone/DNA mass ratio of 9.0; ▼, chromatin templates with a core histone/DNA mass ratio of 1.6.

We next examined the formation of paranemic joint molecules, precursors to plectonemic structures, which readily accumulate in the absence of a free-end in the region of homology (31, 32). We incubated chromatin templates (Fig. 1, substrate b) containing a core histone/DNA mass ratio of 0.8 with nucleoprotein filaments of RecA protein–M13 ssDNA and measured the formation of joint molecules as described above. The rate of formation of joint molecules was about the same as seen in Fig. 3 and the extent was >80% (data not shown).

Chromatin Templates Do Not Support Strand Exchange. In view of the occurrence of the uninhibited formation of joint molecules with chromatin templates at two different mass ratios of core histone/DNA tested, we considered the possibility of strand exchange leading to the synthesis of heteroduplex DNA. To study strand exchange between chromatin templates, containing a core histone/DNA mass ratio of 0.8, and nucleoprotein filaments of RecA protein–M13 ssDNA, we employed agarose gel assay (24). As shown in Fig. 4, with naked duplex DNA as the substrate, the formation of form II DNA (nicked closed circular duplex DNA) was evident as early as 15 min with the concomitant conversion of most of the form III DNA (linear duplex DNA) into form II DNA by 60 min. In contrast, for chromatin templates, the formation of form II DNA was suppressed over the same time period. This inhibition of strand exchange with chromatin templates suggested an obstruction to the passage of nucleoprotein filaments. The question arises as to whether nucleosomes are dislodged from the DNA template during the formation of joint molecules. We have established (see Fig. 2*A*) that nucleosomes restrict access of *Hae* III to DNA. By using the same assay, we observed that naked duplex DNA in the presence of nucleoprotein filaments of RecA protein–

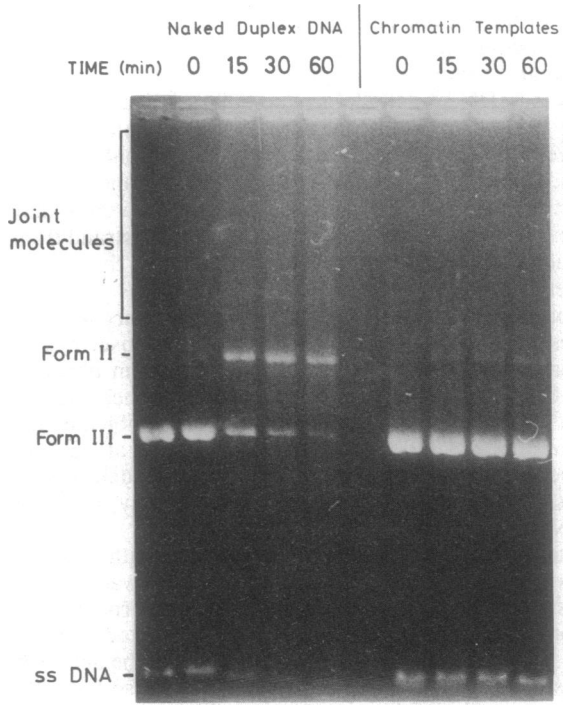

FIG. 4. Reconstituted chromatin templates prevent strand exchange. Reaction mixtures contained standard assay buffer. Samples taken at the indicated time intervals were deproteinized and electrophoresed.

M13 ssDNA was highly sensitive to *Hae* III digestion. Under identical conditions, chromatin templates were protected from *Hae* III digestion (data not shown). Thus we fail to see evidence of nucleosomal disruption during homologous pairing, suggesting that the inhibition of strand exchange observed with chromatin templates was due to the presence of nucleosomes.

Histone H1 Causes Inhibition of Joint Molecules. The failure of core histones to inhibit the formation of joint molecules leaves open the possibility that higher-order structures of nucleosomes do inhibit. Histone H1 is thought to mediate the formation of higher-order structures. Indeed the absence of histone H1 has been invoked to explain the sensitivity of genes being actively transcribed to DNase I (33) and their accessibility to transcription factors (34). A definitive experiment to test this contention should include the addition of histone H1 to chromatin templates prior to the initiation of homologous pairing. In one experiment we added histone H1 to chromatin templates containing a core histone/DNA mass ratio of 1.6, at various stoichiometric amounts. In the other, naked duplex DNA replaced the chromatin templates. Nucleoprotein filaments of RecA protein–M13 ssDNA were then added, and joint molecules were measured after 10 min. As shown in Fig. 5, histone H1 caused complete inhibition at a H1/DNA stoichiometric ratio of 0.1, the amount of H1 associated with chromatin *in vivo*. In contrast, with the same amount of histone H1, the inhibition observed with naked duplex DNA was negligible, however, at a 10-fold excess concentration, it caused inhibition to an extent of 60%.

Lack of Correlation Between the Production of DNA Networks and Formation of Joint Molecules. An impressive correlation has been established between the ability of recombinases, both prokaryotic and eukaryotic, when bound to ssDNA, to mediate the aggregation of naked duplex DNA *in vitro* and the stimulation of the formation of joint molecules (14). The significance of DNA networks *in vivo* is unclear. To obtain insight into this phenomenon, we measured the production of DNA networks with chromatin templates in the presence of nucleoprotein filaments. Consistent with the earlier findings, we observed an excellent correlation between the occurrence of DNA networks and the formation of joint molecules with naked duplex DNA and nucleoprotein filaments (Fig. 6). However, when we switched to chromatin templates with a core histone/DNA mass ratio of 1.6, the DNA networks were produced to an extent of only 20%, whereas the level of formation of joint molecules was indistinguishable from that of the naked duplex DNA. Furthermore, with chromatin templates containing histone/DNA mass ratio of 0.8, virtually no networks were observed but formation of joint molecules was normal (see Fig. 3).

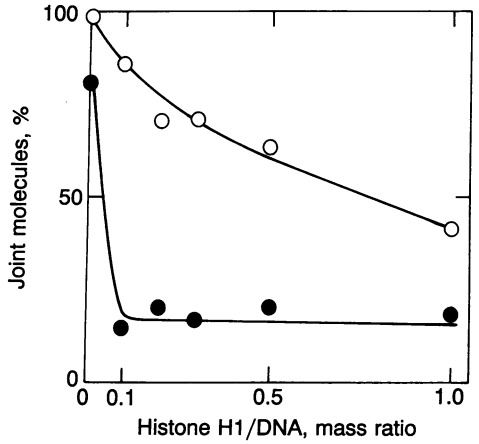

FIG. 5. Inhibition of homologous pairing by histone H1. Reaction mixtures (30 μl) in a standard assay buffer containing 8 μM M13 ssDNA, 1.2 mM $MgCl_2$, and 4 μM RecA protein were preincubated for 10 min at 37°C. $MgCl_2$ to 12 mM and SSB to 0.5 μM were added and mixtures were incubated for an additional 5 min. We then added histone H1, at the indicated concentrations, followed by 5 μM naked duplex [^{3}H]DNA or 5 μM chromatin templates. After taking a sample for the determination of total input radioactivity, we diluted the reaction mixture with 5 ml of ice-cold 1.5 M NaCl/0.15 M sodium citrate and assayed for bound radioactivity as described in Fig. 3. ○, Naked duplex [^{3}H]DNA; ●, chromatin templates with core histone/[^{3}H]DNA mass ratio of 1.6.

DISCUSSION

Most of the molecular models of genetic recombination have invoked a lesion in the DNA, either a single-stranded nick or a double-stranded break, to provoke recombination *in vivo* (35). Genetic studies in a variety of systems as well as *in vitro* reactions have confirmed this prediction (14, 35). Interestingly, the models of genetic recombination do not address the accessibility of DNA sequences in the chromatin to the multienzymatic machinery of homologous recombination. In the light of the foregoing observations, we suggest that attempts to formulate models of genetic recombination and understanding of gene targeting, at the molecular level, should consider DNA in the context of nucleosomes and various degrees of higher-order nucleoprotein structures.

Several observations have indicated that chromatin plays a prominent role in regulating expression of the information in the genome, but virtually nothing is known about the ability of chromatin to serve as a template in homologous recombination. Accordingly, we monitored for homologous pairing between reconstituted chromatin templates, believed to represent the *in vivo* state at the basic level, and nucleoprotein filaments of RecA protein ssDNA. The data presented herein show that homologous pairing was unaffected. The initial rates and the extent of formation of joint molecules were indistinguishable from those of the naked duplex DNA. It is interesting that the core histone/DNA mass ratio of 0.8, which approximately corresponds to the *in vivo* complement of core histones or twice its amount did not suppress homologous pairing. However, complete inhibition of the formation of joint molecules occurred with a 7-fold increase in the

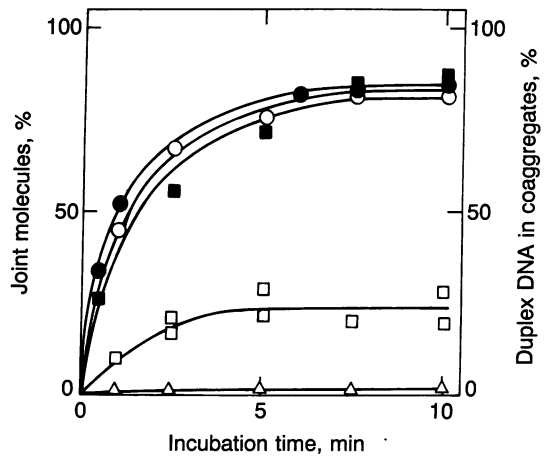

FIG. 6. Absence of correlation between the production of DNA networks and the formation of joint molecules. Reaction mixtures (30 μl) were prepared and reactions were started by the addition of either naked duplex [^{3}H]DNA or reconstituted chromatin templates with various core histone/[^{3}H]DNA mass ratios. Open symbols represent coaggregate formation: ○, naked duplex [^{3}H]DNA; △, chromatin templates with core histone/[^{3}H]DNA mass ratio of 0.8; □, chromatin templates with core histone/[^{3}H]DNA mass ratio of 1.6. Solid symbols show the formation of joint molecules; ●, naked duplex [^{3}H]DNA; ■, chromatin templates with a core histone/[^{3}H]DNA mass ratio of 1.6.

nucleosomal density. Similarly, Knezetic and Luse (36) observed the loss of transcription when the density of nucleosomes was greater than two-thirds of that occurring *in vivo*. Relevant studies have also shown that transcription factors influence the assembly of chromatin templates (37). The nucleosomes assembled on viral promoters in one study (38) inhibited initiation of transcription by SP6 RNA polymerase and in another (39) reduced the efficiency of initiation, but in neither did it impede transcriptional elongation.

Whereas homologous pairing was normal with chromatin templates having a core histone/DNA mass ratio of 0.8 and 1.6, strand exchange was sharply repressed. If such be the case, we wonder how strand exchange is facilitated *in vivo*? In bacteriophage T4 a single stationary molecule of RNA polymerase stalls the movement of a replication fork. This barrier to fork movement is removed by the T4-encoded DNA helicase that dislodges the stationary RNA polymerase (40). It seems reasonable to speculate that a similar mechanism operates to promote strand exchange *in vivo*.

The studies of Conley and West (41) and the experiments reported here on the colligative behavior of RecA protein seem to differ from a number of earlier observations (14). The coaggregates produced by recombinases have been conceived as instrumental intermediates in homologous pairing (14). Coaggregation is not confined to recombinases alone. Agents as diverse as polyethylene glycol, polyamines, histones mixed in low-salt buffers (38), and yeast SSB (28) have similar effects. Nonetheless, conceptually our data (Fig. 6) are not directly in conflict with earlier observations (14). The binding of histones to duplex DNA perhaps reduces the effective search volume and hence coaggregation observed *in vitro* with recombinases has no apparent significance to the pairing reaction *in vivo*.

Genetic studies have indicated correlation among chromatin structure, transcriptional activity, and recombination. For instance, in the mating type interconversion of *Saccharomyces cerevisiae*, the actively transcribed *MAT* locus is cleaved by the HO endonuclease prior to initiation of recombination. The nontranscribed *HML* and *HMR* loci are not cleaved (42). Thomas and Rothstein (43) obtained a 15-fold increase in recombination between direct repeats of the *GAL10* gene associated with an increase in transcriptional activity. In mammalian cells, the expression of the variable gene segments in B cells is closely linked to immunoglobulin gene rearrangements (44). Our experiments show that the chromatin assembled at a histone/DNA mass ratio of 0.8 or 1.6 was receptive to homologous pairing but at a ratio of 9.0 was refractory. However, at all the mass ratios of histones, strand exchange was prevented. This might correlate with the differences observed in the occurrence of recombinants in transformation experiments (2). Furthermore, the events responsible for making microinjected DNA molecules refractory to recombination occur coincidentally with the packaging of the DNA into chromatin (10). Thus our results initiate an understanding of the involvement of chromatin structure in homologous recombination.

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