DNA Ligase Activity on Chromatin and Its Analogs. Rejoining of DNA Strands in Polylysine–DNA Complexes and in Reconstituted Chromatins†

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ABSTRACT: A highly purified DNA ligase from rat liver nuclei has been tested on DNA containing single-strand breaks ("nicks"); the DNA was present in several types of complexes which were chosen to serve as models for chromatin. These model systems included complexes of polylysine or histones with DNA as well as reconstituted chromatin preparations. In all these cases, the limit of ligase sealing was measured as a function of the ratio of polypeptide or protein to DNA. With an excess of either polylysine or histones, the ligase is totally prevented from sealing nicks in the DNA. However, at ratios of histones to DNA similar to those occurring in chromatin, about half of the nicks are accessible to the ligase. In the reconstitution of chromatin, the proteins are dissociated from the DNA by exposure to high ionic strength either with or without urea. If such procedures are carried out in the presence of labeled nicked DNA, the proteins will redistribute over this ligase substrate as well. When the chromatin is reconstituted at protein/DNA ratios similar to those occurring in chromatin, once more only about half of the nicks are accessible to the ligase. Similar results were obtained with preparations reconstituted with either rat liver or duck reticulocyte chromatin. The rate of ligase action has also been measured on a variety of the complexes. While the rate falls as the DNA is increasingly covered with polylysine or histones, this is largely or entirely due to the decrease in concentration of sealable sites. At saturating concentrations of these DNA complexes, the original rate on uncovered DNA is approached.

The DNA of eukaryotes generally exists in the form of deoxynucleoprotein complexes rather than as free DNA. Enzymes which metabolize DNA in eukaryotes are therefore subject to the restrictions and alterations imposed by this complex form of DNA. We have previously described the extensive purification and partial characterization of a DNA ligase from the nuclei of rat liver (Zimmerman and Levin, 1975a). We here extend the study of this enzyme to several types of DNA–protein complexes chosen to serve as models for deoxynucleoproteins. The DNA substrates tested vary from polylysine–DNA complexes and histone–DNA complexes to reconstituted chromatin. In all cases, we followed the sealing of ^{32}P-labeled single-strand interruptions ("nicks"[1]) (Figure 1). The general conclusion from the experiments with the polylysine–DNA complexes and histone–DNA complexes is that potential sites of ligase action which are covered by polylysine or histones are not sealed efficiently by the ligase. Since the limit of ligase action on reconstituted chromatin was found to be about half of the potential number of sites of ligase action on the DNA in this material, reconstituted chromatin behaves as if half of its DNA is covered by materials which are not readily dislodged during ligase action, presumably proteins, while half of the DNA behaves as if it were freely available to the ligase.

Materials and Methods

DNA ligase was purified from rat liver nuclei; enzyme fraction IV stabilized with plasma albumin was used for all measurements. The ligase incubation medium was as previously described and the total concentration of DNA—either in free or complexed form—was maintained at 10 μg/ml except where noted. The extent of sealing of the DNA was estimated by conversion of 5′-$^{32}PO_4$-labeled nicks (one nick per 2000 base pairs in the [^{32}P]DNA) into a form resistant to phosphatase. All of the four common bases occur on both the 5′-phosphate and 3′-hydroxyl sides of the nicks. Details of these procedures have been described (Zimmerman and Levin, 1975a). Where polylysine or histone was added directly to ligase assay mixtures, attempts were made to prevent local excesses. Dilute solutions of these materials were added with constant mixing to chilled tubes containing the remainder of the assay mixtures complete except for enzyme. After 10 min on ice, ligase was added followed by incubation at 37°. At the conclusion of the ligase incubation, polylysine or histones were added to bring the mixtures to 1 or 6 μg, respectively, in these materials before acid precipitation. Sealing was estimated as above. The *limit* of ligase sealing on a particular sample of a DNA or DNA complex was obtained from separate incubations containing 0, 1, 2, or 4 units of ligase. The ^{32}P sealed in the presence of the ligase was corrected for a small blank in its absence (<1% of the acid-precipitable ^{32}P). These levels of ligase routinely gave amounts of sealing varying by <10%, indicating that the maximal extent of sealing had occurred. More elaborate series of kinetic measurements and measurements in which the levels of enzyme or DNA were varied considerably indicated that such values did indeed represent limits of ligase action. Total acid-precipitable ^{32}P was also estimated for all the DNA samples (phosphatase treatment omitted in the assay protocol); this value was not influenced by the presence of the ligase during the incubation. Several preparations of ^{32}P-la-

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[1] The following terminology is used: nicked DNA is DNA containing single-strand breaks; a single-strand break is used to indicate an interruption of one of the two strands of bihelical DNA which is bordered by a 5′-phosphate and a 3′-hydroxyl group.

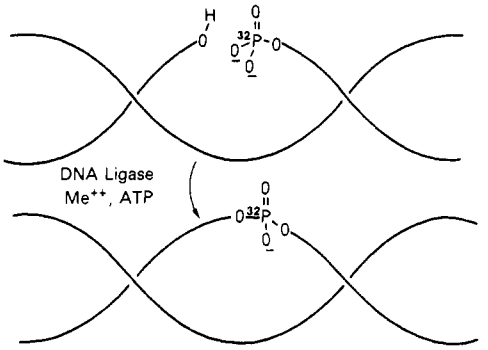

FIGURE 1: DNA ligase sealing of a ^{32}P-labeled nick in bihelical DNA.

beled nicked DNA prepared according to Zimmerman and Levin (1975a) were used in the course of this work. The ligase sealed from 66 to 72% of the acid-precipitable ^{32}P in these preparations. The remaining ^{32}P was presumably located at double-stranded ends or where gaps prevented closure of single-strand breaks. All of the limits on DNA complexes are expressed as fractions of the limit of sealing on the uncomplexed [^{32}P]DNA.

Solubility of [^{32}P]DNA in Polylysine Complexes or in Reconstituted Chromatins. Assay tubes were composed exactly as those used to measure ligase action. After incubation at 37°, the tubes were immediately centrifuged for 5 min at room temperature at 8000*g*. The supernatant fluids were removed with a Pasteur pipet. The supernatant fluids and redissolved pellets were assayed either directly for acid-precipitable radioactivity, or for acid-precipitable radioactivity after phosphatase treatment (as in the ligase assay procedure) to determine the distribution of the ligase product.

Nuclease Sensitivity of the [^{32}P]DNA in Complexes with Polylysine. After completion of the incubation as for the ligase assay, either pancreatic DNase or staphylococcal nuclease (Worthington) was added and incubation continued for 5 min at 37°. Carrier was added and radioactivity measured after precipitating twice with trichloroacetic acid as in the final steps of the ligase assay.

Chromatin Isolation. Nuclei were purified from rat liver (Zimmerman and Levin, 1975a) and chromatin isolated by essentially the procedure of Axel et al. (1973) omitting the detergent treatment of the nuclei. The content of protein (plasma albumin standard) and RNA (alkaline hydrolysis) was 2.0 and 0.1 mg, respectively, per mg of DNA (based on total phosphate). Duck reticulocyte chromatin (Axel et al., 1973) was a gift of G. Felsenfeld.

All chromatin preparations were sheared before use (Virtis homogenizer, 2 min at 90 V).

Reconstitution of Chromatin-DNA or Histone-DNA Mixtures. Reconstitution of chromatin or histones with DNA was generally carried out by exposure of the mixtures to a concentrated salt and urea solution followed by a series of dialyses to remove the salt in a stepwise fashion and finally to remove the urea. This procedure is detailed below; some alternative procedures are listed in Table I.

For stepwise salt-urea reconstitutions, mixtures were made with varying proportions of chromatin and ^{32}P-labeled nicked DNA (total DNA-P concentration, 1.6 μmol in 2.0 ml of 0.02 *M* β-mercaptoethanol-0.1 m*M* EDTA). The procedure for reconstitution of this mixture is slightly modified from the protocol of Axel et al. (1974). NaCl (350 mg) and urea (900 mg of Schwarz/Mann "ultrapure" grade) were dissolved at 0° in the mixture of chromatin and DNA. The final volume was 2.7 ml corresponding to 2.2 *M* NaCl and 5.6 *M* urea. After 1 hr at 0°, the mixture was dialyzed at 5° for 1-hr periods against 200-volumes of 5 *M* urea-0.02 *M* β-mercaptoethanol containing 1.2, 1.0, 0.6, and finally 0 *M* NaCl. The samples were then dialyzed against two changes of 0.1 m*M* EDTA for 1 and 16 hr, respectively. The endogenous DNA ligase activity present in the chromatin preparations was irreversibly inactivated by this reconstitution procedure. The DNA was reisolated from many of the reconstituted chromatin preparations by extraction with freshly neutralized redistilled phenol in the presence of 1 *M* NaCl followed by dialysis against 1 m*M* Tris-HCl buffer (pH 8.0) containing 0.1 m*M* EDTA.

Table I: Effect of Different Reconstitution Procedures upon the Extent of Ligase Sealing of Nicked DNA in Reconstituted Rat Liver Chromatins.

Reconstitution Procedure	$P_{chromatin}/(P_{chromatin} + P[^{32}P]DNA)$	Limit of Ligase Sealing[a]
1. Dissociation in 2.2 *M* NaCl–5.6 *M* urea–0.02 *M* β-mercaptoethanol; reduction of NaCl in four steps by dialyses in the presence of urea and mercaptoethanol; dialyze out urea (see Methods for details)	0.89	0.49
2. As in 1 but mercaptoethanol omitted[b]	0.90 0.97	0.51 0.45
3. As in 1 but urea omitted	0.91	0.45
4. Dissociation in 2 *M* NaCl and 0.02 *M* β-mercaptoethanol; one-step reduction of NaCl by dialysis against 0.1 m*M* EDTA	0.90 0.96	0.42 0.40
5. As in 4 but reduce to 0.6 *M* NaCl by rapid dilution with water	0.96	0.32
6. As in 4 but reduce to 0.15 *M* NaCl by rapid dilution with water	0.96	0.34

[a] Expressed as the fraction of the total sealable nicks in the [^{32}P]-DNA used in the reconstitution protocols. [b] Histone F3 was mainly present as the dimer.

Histones and Polylysine. Histones were extracted from rat liver chromatin with multiple aliquots of cold 0.4 *N* H_2SO_4, precipitated with cold 3.1 *M* trichloroacetic acid, and washed with diethyl ether. The yield corresponded to 1.28 mg of histone/mg of DNA which will be assumed to be the average in vivo histone content of our chromatin preparations for the experiments in which these acid-extracted histones were added back in varying proportions to [^{32}P]DNA under reconstitution conditions. A commercial sample of histones from calf thymus (Worthington) was used in certain experiments. Polylysine hydrobromide was obtained from Miles-Yeda (molecular weight range, 30,000–70,000). The concentration of the stock solution was determined by the Kjeldahl nitrogen procedure (N.I.H. Microanalytical Laboratory).

Other Methods. Protein was determined according to Lowry et al. (1951) with bovine plasma albumin as a standard. Total phosphate was estimated according to Ames and Dubin (1960). Radioactivity was measured with a low-background Geiger counter (Tracerlab Omni/Guard).

Results

Polylysine-DNA Complexes

At low ionic strengths polylysine has been shown to bind

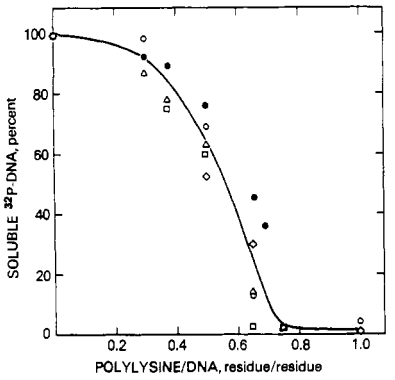

FIGURE 2: Solubility of DNA in mixtures of ^{32}P-labeled DNA with polylysine under DNA ligase assay conditions. Different symbols indicate independent experiments.

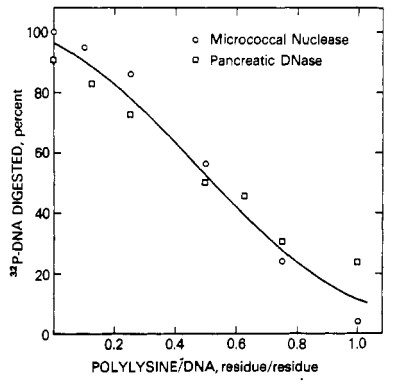

FIGURE 3: Nuclease digestion of ^{32}P-labeled DNA in mixtures of ^{32}P-labeled DNA with polylysine under DNA ligase assay conditions. Pancreatic DNase (25 μg) or staphylococcal (micrococcal) nuclease (10 μg) was added where indicated; one-third the level of either of these enzymes gave the same amount of acid-soluble ^{32}P.

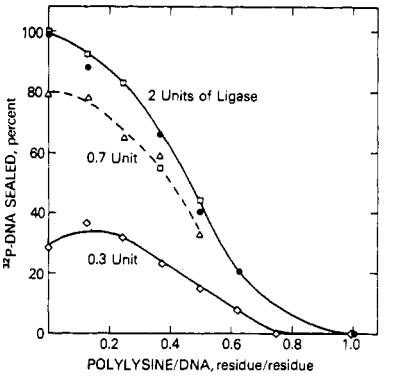

FIGURE 4: DNA ligase activity on polylysine–DNA complexes.

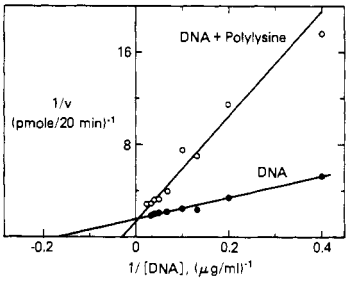

FIGURE 5: DNA concentration effect on the rate of ligase sealing of nicked DNA or nicked DNA partially covered with polylysine. Where indicated, the polylysine was present at a ratio of 0.5 residue/DNA residue.

to DNA in an essentially random and irreversible manner (Tsuboi et al., 1966). We will show similar behavior under ligase assay conditions based upon solubility properties and the nuclease sensitivity of the complexes. The complexes were tested as substrates for ligase.

Solubility of Polylysine–DNA Complexes. A series of complexes were formed having increasing proportions of polylysine to DNA up to one polylysine residue per DNA phosphate. After incubation under the ligase assay conditions, the mixtures were centrifuged. As polylysine is added, the DNA becomes insoluble (Figure 2). As the ratio of polylysine to DNA increases, the fraction of insoluble ^{32}P increases slowly and then rather abruptly all of the ^{32}P becomes insoluble. This behavior is consistent with random binding of polylysine to DNA followed by precipitation when the degree of charge neutralization is sufficient. Cooperative binding of polylysine has been shown (Olins et al., 1967; Shapiro et al., 1969) at much higher ionic strengths and results in essentially a direct proportionality between the amount of polylysine added and the amount of DNA precipitated.

Digestion of Polylysine–DNA Complexes with Pancreatic DNase or Staphylococcal Nuclease. After incubation under the ligase assay conditions for 20 min at 37° relatively large amounts of either pancreatic DNase or staphylococcal nuclease were added and the incubation continued for 5 min. With either nuclease the extent of digestion did not change significantly as the nuclease concentration was varied several-fold, indicating that digestion was proceeding to a limit under these conditions. The amount of [^{32}P]DNA resistant to the nucleases increased essentially linearly with the ratio of polylysine to DNA; at one polylysine residue for each DNA phosphate, the DNA had become almost totally resistant to digestion (Figure 3). The reasonably well-defined limits indicate polylysine binding is essentially irreversible under these conditions, and that protection of DNA from the nucleases proceeds with little or no lag as polylysine is added. In particular, the protection is not well correlated with the solubility changes outlined above.

Ligase Action on Polylysine–DNA Complexes. Both the *maximum amount* of sealing attained with large amounts of ligase as well as the rate of sealing with lower amounts of ligase decrease as more polylysine is added to a fixed amount of DNA (Figure 4). The shapes of these curves are similar. The decline in the *rate* of sealing seems to be a direct result of the decrease in the *concentration* of sealable nicks as they are covered by polylysine as shown by the following experiment. As the concentration of a polylysine–DNA complex (0.5 residue of polylysine/DNA residue) was increased, the rate of sealing also increased and an extrapolation to saturating conditions indicated there was little or no difference in the maximal ligase rate on DNA sites on either free DNA or on DNA half-covered with polylysine (Figure 5). The decrease in the ligase *limit* is most simply correlated with the decrease in the number of sites as they are covered by polylysine, as shown by the similar polylysine dependence of the limits of nuclease and ligase (Figures 3 and 4, respectively).

As the ratio of polylysine to DNA increases, the fraction of the DNA which is readily sedimentable increases, as mentioned above. When such partially insoluble mixtures are used as the substrate for the ligase, a significant fraction of the product is found in the insoluble fraction (Figure 6). The location of the product in the insoluble fraction does

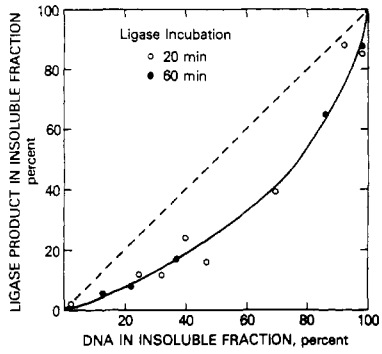

FIGURE 6: Fraction of the [^{32}P]DNA sealed by the ligase found in an insoluble form. One unit of ligase was present in each reaction mixture.

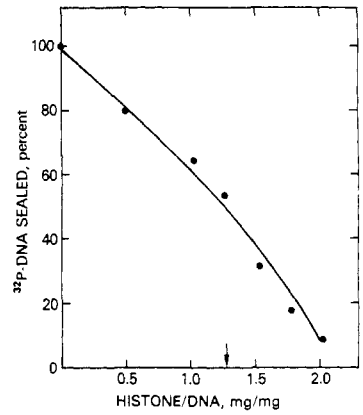

FIGURE 7: Proportion of sealable nicks in DNA in DNA–histone mixtures. The arrow on the abscissa indicates the average in vivo ratio of histone to DNA based upon total acid-extractable protein as described under Materials and Methods.

not of course guarantee that ligase acted upon the DNA while it was insoluble; however, if the product was originally soluble and appeared in the insoluble fraction because of rapid exchange of DNA between soluble and insoluble fractions, the fraction of insoluble ligase product should have been similar to the fraction of insoluble DNA (dashed line, Figure 6). This was clearly not the case. If the exchange of DNA between soluble and insoluble fractions were rate limiting for sealing, the fraction of ligase product that is insoluble should increase with the time of incubation. That was not observed to be the case (Figure 6). The simplest explanation is that ligase can work on DNA in highly aggregated complexes but works at somewhat diminished rates compared to the rate upon DNA in free solution.

The polylysine binding is essentially irreversible in terms of the time scale of the experiments described here (see above). Hence we may ask whether the ligase can work on those DNA sites which are protected from nuclease by polylysine. The ligase product formed on a 0.5:1 polylysine–DNA complex was tested for resistance to staphylococcal nuclease. The limits of ligase sealing and nuclease hydrolysis in this complex were virtually identical (39 vs. 42% of the values in the absence of polylysine). Over 85% of the ligase product was in the fraction sensitive to nuclease digestion, indicating that the ligase and staphylococcal nuclease generally act on the same class of sites with partially covered DNA.

Histone–DNA Complexes

The experiments described above for the polylysine complexes were repeated with complexes formed between DNA and a mixture of total histones from calf thymus. As increasingly larger amounts of histone were added under these conditions, an increasingly larger fraction of the DNA became both insoluble and resistant to nucleases. Coordinately the rate of ligase action decreased. Once again, the V_{max} for a mixture of histone and DNA (1.2 μg of histone/μg of DNA) was similar to that on histone-free DNA. The limit of ligase action reached zero between 1.6 and 2.2 μg of histone/μg of DNA (unpublished experiments of the authors). These experiments, carried out under conditions where there was relatively little opportunity for the histones to assume an equilibrium distribution with relation to themselves and to the DNA, thus yielded qualitatively the same results as did the polylysine experiments.

We then tested the effects of histones when they were allowed to redistribute upon DNA under more nearly equilibrium conditions. Nicked and labeled rat liver DNA was mixed with rat liver histones and then exposed to high salt and urea concentrations which were then subsequently reduced by stepwise dialyses as described under Materials and Methods. As the ratio of histones/DNA was increased in this annealing procedure, the ligase limit on the reconstituted complex fell monotonically (Figure 7). At a ratio of histone/DNA equal to the average ratio in the cell (arrow, Figure 7) the limit had fallen to about 48% of that in the absence of histones. This ratio of histones/DNA assumes quantitative extraction of histones under the conditions used (see Materials and Methods). If extraction were, for example, only 90% complete the ligase limit would be reduced to about 42% at the average cellular ratio of histones/DNA. It should be noted that sufficiently greater levels of histones made the nicked DNA unavailable to the ligase (Figure 7).

Reconstituted Chromatins

We next reconstituted chromatin in the presence of the ^{32}P-labeled nicked DNA substrate. There were two reasons for examining such DNA complexes in addition to the histone–DNA complexes discussed above. First, by increasing the proportion of chromatin to [^{32}P]DNA, the level of chromatin-associated materials to which the [^{32}P]DNA is exposed may be made to closely approach—but cannot exceed—the level in normal chromatin; hence, extrapolation of behavior of the [^{32}P]DNA to that of DNA with its full cellular complement of proteins is simplified. Secondly, the influence of components of the chromatin other than those in the acid-extractable or "histone" fraction may be observed.

Comparison of Chromatin with Reconstituted Chromatin. Staphylococcal nuclease digestion of chromatin has a number of distinctive attributes. At low ionic strength, nuclease digestion halts when half of the DNA of the chromatin becomes acid soluble (Clark and Felsenfeld, 1971). The remaining half of the DNA exists as a well-defined series of relatively short DNA pieces (Axel et al., 1974). These characteristics of nuclease digestion were used as a "fingerprint" with which to compare the behavior of both the total DNA and the ^{32}P label in the reconstituted chromatin.

An excess of staphylococcal nuclease rendered 50–60% of the DNA-P of rat liver chromatin and reconstituted chromatin into an acid-soluble form under conditions described by Clark and Felsenfeld. About 85% of the ^{32}P of the reconstituted chromatin was released, presumably a reflection of both a significant amount of label at ends or gaps (ca. 30%

of the ^{32}P based on its inability to be sealed by ligase) which are not blocked by histones and perhaps of a tendency for nicks to be relatively more uncovered to small probes like staphylococcal nuclease than to larger probes like the ligase.

The DNA isolated from the nuclease resistant fractions of either chromatin or reconstituted chromatin showed identical molecular weight patterns on gel electrophoresis; the band patterns were similar though not identical with those of duck reticulocyte chromatin (Axel et al., 1974). An autoradiographic record of the ^{32}P distribution in the gel electrophoresis pattern from the reconstituted chromatin indicated the presence of ^{32}P only in those regions where the DNA bands occurred.[2]

The DNA was reisolated from the reconstituted chromatins in most of the experiments described here. In no case did the fraction of labeled nicks which could be sealed by the ligase differ by >10% between the reisolated DNA and the original labeled nicked DNA added at the time of reconstitution. Histones extracted from reconstituted chromatins showed an identical pattern on gel electrophoresis (Panyim and Chalkley, 1969) to those from the original chromatin.

The Limit of Ligase Action on Reconstituted Chromatins. The ligase reaction reached well-defined limits[3] on such reconstituted preparations. The limit was independent of the amount of ligase added and the incubation period. While the assay conditions are largely dictated by the requirements of the ligase for a suitable ionic strength and divalent cation concentration, those relatively small variations in the assay conditions which still provided sufficient enzyme activity to approach definite limits caused no significant change in the limits observed.[4]

The input ratio of chromatin/[^{32}P]DNA had a striking effect on the ligase limit of the reconstituted products. The limit on the [^{32}P]DNA fell linearly as the [^{32}P]DNA became a smaller part of the total input (Figure 8). This decrease is, of course, expected from results of the histone titrations of [^{32}P]DNA (Figure 7), and is presumably largely or entirely due to redistribution of histones from the chromatin to the [^{32}P]DNA. A similar decline in limit was seen using chromatin from a heterologous source, duck reticulocytes (Figure 8). In both experiments about 40% of the potentially sealable nicks in the [^{32}P]DNA were sealed at high ratios of chromatin/[^{32}P]DNA. A similar value was found using quite different regimens for the reconstitution, i.e., dissociation by 2 *M* NaCl without urea followed either by relatively gradual reduction or by very rapid reduction of salt concentrations (Table I).

Solubility of Reconstituted Chromatins under Ligase Assay Conditions. As the input ratio of chromatin/[^{32}P]DNA increases, the reconstituted product is increasingly insoluble under conditions used to assay ligase (Figure 8). At high ratios, about 90% of the label is readily sedimentable. There is no obvious correlation between changes in solubility and in ligase limits.

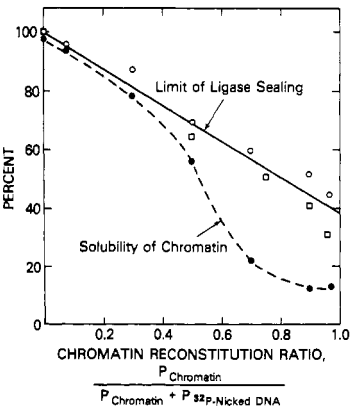

FIGURE 8: Proportion of DNA nicks which can be sealed by DNA ligase and solubility of chromatin in reconstituted chromatins prepared at different ratios of chromatin components to nicked DNA. Ligase limit on reconstituted rat liver chromatin (○) and reconstituted duck reticulocyte chromatin (□); solubility of reconstituted rat liver chromatin (●).

Migration of Chromatin Materials from Chromatin to DNA under Ligase Assay Conditions. If chromatin is added to the [^{32}P]DNA in the assay mixture, the ligase limit on the latter is slightly reduced; e.g., at (chromatin DNA)/([^{32}P]DNA) = 8, the limit on the [^{32}P]DNA is reduced ca. 12%. If the [^{32}P]DNA is preincubated[5] for an hour with this amount of chromatin, the limit upon subsequent incubation with ligase is reduced ca. 35%. The reconciliation of this facile migration of chromatin materials onto the [^{32}P]DNA with a ligase limit very far from complete sealing in reconstituted chromatin will be considered in the Discussion.

Discussion

We first tested the ability of ligase to act on complexes of DNA partially covered with polylysine. The ligase acted with high efficiency (undiminished V_{max}) on those regions uncovered by polylysine but was unable to displace the polylysine at a significant rate. This behavior was true irrespective of the precipitation of the DNA at higher extents of covering. The ligase limit was similar to that for nuclease digestion at varying extents of covering by polylysine. Encouraged by this relatively simple type of behavior as well as by generally similar results with a mixture of histones, we tested the ligase on several types of artificial or reconstituted chromatins. In one type, the acid-extractable ("histone") fraction of chromatin was allowed to redistribute over nicked DNA. Given sufficient amounts of histones, the nicks were made unavailable to ligase; however, given the level of histone to DNA which is the average in vivo, about half of the nicks remained accessible to the ligase. Alternatively, we mixed chromatin with nicked DNA in varying proportions and allowed the chromatin components to redistribute over both the DNA in the chromatin and the nicked DNA. As the ratio of chromatin to nicked DNA was increased, the limit of the ligase on the nicked DNA decreased, extrapolating again to about half of the nicks being accessible to the ligase. The proportion of DNA accessible to the ligase in these chromatin models is thus similar to the proportion of chromatin accessible to some other probes, in-

[2] B. Sollner-Webb, unpublished observations.

[3] The *rate* of the ligase upon reconstituted chromatin preparations varied <twofold from that on the corresponding DNA reisolated from these preparations.

[4] The limit of ligase sealing on a reconstituted rat liver chromatin preparation did not change significantly (48 vs. 50%) when the NaCl concentration was reduced from 0.08 to 0.045 *M* (both in the presence of 2 m*M* $MgCl_2$); in the presence of 0.01 *M* $MgCl_2$ and no NaCl or 1 m*M* $MnCl_2$ and 0.08 *M* NaCl or 2 m*M* $MgCl_2$ and 0.18 *M* NaCl, over 35% of the nicks were sealed.

[5] The preincubation was done in the absence of ATP to prevent sealing by the endogenous DNA ligase activity in the chromatin.

cluding several small molecules or ions, polypeptides, or enzymes. There are, however, examples of probes which act either to greater or to lesser extents on chromatin. These results have been reviewed (Phillips, 1971; Fredericq, 1971; Huberman, 1973; Simpson, 1973) and are further discussed by Itzhaki (1974) and by Clark and Felsenfeld (1974).

It is obviously of paramount importance to know how closely our models for chromatin approach the real structure of chromatin. The polylysine–DNA complexes are models for partial covering and charge neutralization of DNA phosphate groups. There is, however, evidence (Carroll and Botchan, 1972; Li et al. 1973) that polylysine binds in a distinctively different manner from the histones. The structure of chromatin is presently uncertain; hence it is difficult to assess the reconstitution procedure except by comparison of the product with certain distinctive properties of chromatin per se. Several studies (Paul and More, 1972; Axel et al., 1973, 1974) have indicated that such reconstituted chromatin preparations share a number of important properties of chromatin including specificity for transcription. The staphylococcal nuclease digestion of chromatin yields half of the DNA in a discrete set of molecular weight species (Axel et al., 1974). Our labeled preparations of reconstituted chromatin not only yield such bands but contain the label distributed throughout the region of the bands. Even given a similar average structure for chromatin in these reconstituted preparations, it is clear that nicks in DNA may have special attributes. Hence, using a probe such as the DNA ligase which probably reports primarily on the locale of the nicks may give an answer not representative of the bulk of the chromatin but perhaps of special interest in view of the use of nicks as initiation points for in vitro RNA synthesis by mammalian RNA polymerases (Meilhac and Chambon, 1973).

What are the in vivo implications of the limited accessibility of nicks to the ligase in vitro? A direct extrapolation would suggest that nicks in histone-bound regions of DNA would persist unsealed. Indeed, single-strand breaks do accumulate with age in rat liver DNA (Massie et al., 1972), but it is unknown both whether these breaks have the proper structure to be sealed by the ligase and whether the breaks occur in ligase-accessible or -inaccessible fractions of the DNA. In contrast, several recent reports are consistent with some repair of DNA damage in histone-covered regions. For example, Paterson et al. (1973) found about half of the uv-induced thymine dimers are removed at a much slower rate than the rest; those authors suggested that either inaccessibility to excision repair or a slower, postreplication repair could explain their data. Wilkins and Hart (1974) found about half of the thymine dimers are initially covered by proteins, and the dimers in this covered fraction decrease relatively slowly. Finally, Lieberman and Poirier (1974) demonstrated repair synthesis in mouse satellite DNA in the absence of DNA replication; the satellite DNA is heterochromatic and apparently not translated. These results suggest that in vivo there may be a "review" of chromatin DNA to repair defects in histone-bound regions. Frenster (1965) proposed that polyanions might free covered DNA from histones by direct competition. Model studies have demonstrated this and related effects (Jensen and Chalkley, 1968; Ilyin et al., 1971; Arnold et al., 1972). Similar competition in vivo could possibly mediate repair in histone-covered regions. Such repair might be of considerable importance in vivo; the accumulation of damage in DNA between rounds of replication has been postulated to be involved in a variety of cellular malfunctions ranging from aging to malignancy. The experimental basis and implications of this hypothesis have recently been discussed by Yielding (1974).

We have used the limited ability of the ligase to seal nicks in reconstituted chromatins to demonstrate the lack of DNA sequence specificity for histone binding in reconstituted chromatins (Zimmerman and Levin, 1975b). A single round of reconstitution of ^{32}P-labeled DNA ligase substrate with a large excess of chromatin followed by ligase sealing produces a reconstituted chromatin in which about half of the DNA nicks are sealed. Repeating the reconstitution procedure (without further addition of chromatin) followed again by ligase sealing results in an increase in the total amount of sealing to the level predicted for a complete lack of DNA sequence specificity in the binding of histones to the DNA. In this experiment, we used homologous DNA and proteins and asked whether there is a limited number of preferred sequences to which histones will bind. In other words, we offered the histones two chances to bind to the DNA; no correlation was found between the sites the histones picked each time. This experiment clearly indicates that there is an excess of sites available in the chromatin for binding of histones at the in vivo ratio of histones to DNA. Exchange of histones between these sites apparently does not readily occur under ligase assay conditions or else all of the nicks would eventually be exposed and become sealed, as opposed to the observed limit of sealing of half of the nicks. It is noteworthy, then, that the histones readily transfer to exogenous [^{32}P]DNA under these conditions. This result implies that the unfilled histone-binding sites on the chromatin are distinctly different from those on histone-free exogeneous DNA, in accord with the earlier similar conclusion of Ilyin et al. (1971).

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Terbium as a Fluorescent Probe for DNA and Chromatin†

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ABSTRACT: Terbium reacted with DNA and chromatin to form a complex in which terbium acted as a sensitive fluorescent probe. By measuring the narrow-line emission of Tb^{3+} when DNA is selectively excited, the relative amount of Tb^{3+} bound to the DNA can be calculated. Terbium was bound to DNA until one Tb^{3+} was present for each phosphate group. After this point no more terbium was bound. $TbCl_3$ was bound to chromatin in a linear manner until approximately 0.48 $TbCl_3$ was added for each phosphate group in the chromatin-DNA solution. From these data it appears that 52% of the phosphate groups in chromatin were unavailable for binding. The binding of Tb^{3+} to DNA can be reversed by prolonged dialysis against 0.5 *M* NaCl and chelating agents. The terbium ion is ideal in that it binds DNA tight enough so that completion of the reaction can be assumed but loose enough so that it can be removed by gentle means. Low concentrations of salt (up to 2 m*M* NaCl) enhance the quantum efficiency. Below pH 3 and above pH 7 the DNA-terbium complex will not form. Between pH 3 and pH 7 the quantum efficiency of the DNA terbium complex increases from either pH to a maximum at pH 5.5 to 5.6. Several biochemical uses for Tb^{3+} ion are suggested.

The use of fluorescent probes in the development of methods which yield information about the nature of nucleic acid and chromatin is an important focus in biochemistry today. Organic molecules have been used as fluorescent probes with nucleic acid and chromatin, most notably ethidium bromide and several acridine dyes (Blake and Peacocke, 1968; LePecq and Paoletti, 1967). Several modes of binding can occur with these dyes ("strong and weak", "intercalated or external binding") depending upon the ratio of dye to DNA-P, the ionic strength of the media, and the pH (von Hippel and McGhee, 1972). Several quantitative methods for determining free phosphate in chromatin using Toluidine Blue and azure A have been reported (Klein and Szirmai, 1963; Miura and Ohba, 1967). More recently Clark and Felsenfeld (1971) and Itzhaki (1970) have used polylysine to titrate the "open" portions of chromatin.

Inorganic fluorescent probes, especially the lanthanide ions, have been an area of intense research interest; since Heller and Wasserman (1965) found that selective excitation of the ligands of rare earth chelates yields characteristic line emissions of the lanthanide ions. Crosby (1966) showed that the ligand triplet state must be higher than the resonance level of the lanthanide ion for effective transfer to occur. Filipescu and Mushrush (1968) suggested the use of unchelated lanthanide ions as fluorescent probes for excited triplets in solution. Eisinger and Lamola (1971) published a kinetic model for the transfer of energy from common nucleotides to lanthanide ions in aqueous solution at pH 5.0 or less so that no association with phosphate groups occurred. Luk (1971) had used the lanthanide ions as fluorescent probes in the study of the transferrin molecule conformation. Based on fluorescence, two specific binding sites per transferrin molecule were found.

This paper reports the use of Tb^{3+} as a sensitive fluorescent probe in the solid state with DNA and chromatin. At neutral or slightly acidic pH's terbium will precipitate DNA and chromatin. By measuring the narrow line emission of Tb^{3+} when the DNA is selectively excited, the relative amount of Tb^{3+} bound to the DNA can be calculated.

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