Interaction of a Reporter Molecule with Chromatin. Evidence Suggesting That the Proteins of Chromatin Do Not Occupy the Minor Groove of Deoxyribonucleic Acid*

Robert T. Simpson

ABSTRACT: The interaction of the reporter molecule, 2,4-$(NO_2)_2C_6H_5NH(CH_2)_2N^+(CH_3)_2(CH_2)_3N^+(CH_3)_3 \cdot 2Br^-$, with chromatin of rabbit liver has been studied. Gabbay and coworkers have previously shown that this reporter binds to adjacent phosphate anions of nucleic acids, inducing hypochromism and circular dichroic absorption in the spectrum of the bound reporter molecule, and have suggested that binding occurs in the minor groove of the DNA double helix.

The present paper shows that the reporter binds to chromatin without dissociation of the proteins of the complex. No qualitative differences in the mode of reporter binding to DNA and to chromatin were detected by circular dichroism measurements. The number of reporter binding sites (1 for each 6 DNA phosphates) was found identical for free DNA and for DNA complexed in chromatin, and the stability of reporter binding was nearly similar in the two instances. These results suggest that the proteins of chromatin occupy only the major groove of the DNA double helix, leaving the minor groove available for interaction with the reporter, or with other physiologically significant small molecules in the cell nucleus.

The detailed macromolecular architecture of chromatin[1] has remained unsolved, despite a number of attempts at its elucidation. X-Ray diffraction studies have been of some success in analyzing the structure of DNA in chromatin, but have added little information on the structure of the proteins of chromatin, or the types of interaction between these two major components of the chromatin complex (Wilkins *et al.*, 1959). Previous investigations of the spectroscopic and hydrodynamic properties of chromatin have also generated insight into the structure of DNA and protein in this species. These studies have suggested that the DNA of chromatin is shortened (Ohba, 1966), and probably partially unstacked relative to DNA in solution (Tuan and Bonner, 1969; Permogorov *et al.*, 1970; Simpson and Sober, 1970).

The proteins of nucleohistone are also altered in conformation relative to their structure when free in solution, becoming more α helical when complexed with DNA (Zubay and Doty, 1959; Tuan and Bonner, 1969; Permogorov *et al.*, 1970; Shih and Fasman, 1970; Simpson and Sober, 1970). The slightly lysine-rich histone fraction appears to be involved in constraint of the DNA into its altered conformation in chromatin (Tuan and Bonner, 1969; Simpson and Sober, 1970). Studies of the melting properties of chromatin have suggested that histone clusters of a single type do not occur in nucleohistone (Ohlenbusch *et al.*, 1967). In all these studies little information concerning the specific interactions of proteins and DNA in chromatin has been engendered.

Detailed information on the structure of chromatin is required in order to understand the regulation of transcription, and the control of cellular development and differentiation. The current studies have approached the structural properties of chromatin by examination of the binding of a reporter molecule. The reporter, *N,N,N*-trimethyl-N^1,N^1-dimethyl-N^1-(β-2,4-dinitroanilinoethyl)-1,3-diammoniumpropane dibromide, has been previously studied by Gabbay and his coworkers in its binding to DNA, RNA, and synthetic polynucleotides (Gabbay, 1968, 1969; Gabbay and Mitschele, 1969: Gabbay *et al.*, 1969; Passero *et al.*, 1970). The present studies show that the mode of binding of the reporter to both free DNA and to chromatin DNA appears similar; that binding of the reporter to chromatin does not lead to dissociation of the protein components of this complex; and that the number of binding sites for the reporter does not differ for DNA free in solution *vs.* DNA complexed in chromatin.

Experimental Section

Chromatin was isolated from mature female rabbit liver using the methodology previously described for calf liver (Simpson and Sober, 1970). Nuclei were initially isolated using the Triton X-100 procedure of Hymer and Kuff (1964). Chromatin was then prepared from the isolated nuclei by repeated sedimentation from media of decreasing ionic strength (0.025–0.0005) at pH 7.8 as described by Huang and Haung (1969). The mass ratio of protein to DNA in such chromatin was 1.9 to 1. As determined from acid solubility (0.2 N HCl, 4°, 20 min) 70% of the protein was histone, and 30% nonhistone protein. DNA was a highly polymerized commercial product obtained from calf thymus (Worthington Biochemicals Corp.).

N,N,N-Trimethyl-N^1,N^1-dimethyl-N^1-(β-2,4-dinitroanilinoethyl)-1,3-diammoniumpropane dibromide, the reporter employed in the studies, was a gift from Dr. Edmund Gabbay.

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[1] The terms chromatin and nucleohistone will be employed interchangeably to describe the sheared soluble form of the interphase chromatin of mammalian cells.

DNA concentrations were determined either by the diphenylamine method (Schneider, 1957), or, alternatively, by measurement of the absorbance at 260 nm of the material solubilized by hydrolysis in 5% perchloric acid for 20 min at 100°, using 30 as the absorbance of a solution of 1 mg/ml of hydrolyzed DNA under these conditions. Protein concentrations were determined by the method of Lowry *et al.* (1951) as described by Layne (1957).

pH measurements were made with a Radiometer PHM 27 meter equipped with glass and calomel electrodes. Absorption measurements were obtained with a Zeiss PMQ II spectrophotometer. Circular dichroism measurements were performed with a Cary Model 60 automatic recording spectropolarimeter equipped with the Model 6002 circular dichroism accessory. Standardization of this instrument, and its examination for potential absorption artefacts, were performed as previously described (Simpson and Sober, 1970). Molecular ellipticities, $(m)_\lambda$, refer to the concentration of reporter.

Gel filtration studies of reporter binding to chromatin were carried out according to the procedure suggested by Hummel and Dreyer (1962). A 1.2 × 25 cm column of Bio-Gel P-60 equilibrated with the reporter in phosphate buffer was employed in all studies. In this and all other studies, the buffer was sodium phosphate, 9×10^{-3} M in sodium ion, pH 7.3. A sample of chromatin in reporter buffer was passed into the column, and washed in with two 0.5-ml portions of the reporter–buffer mixture. Elution was carried out with the same buffer used for equilibration of the column, at a flow rate of 8 ml hr^{-1}. Fractions were collected for measurement of protein content and measurement of absorbance at 345 nm, reflecting the presence of the reporter molecule. In some experiments, absorbance of the column eluate at 345 nm was continuously monitored using a Beckman DB-G spectrophotometer equipped with either a 0.20- or 1.00-cm path-length flow cell and a 10-in. linear-log recorder.

Detailed evaluation of the binding stoichiometry of the reporter to DNA and to chromatin was performed by measurement of the absorption of the complexes at 345 nm. Reporter concentration was maintained constant at 2.5×10^{-5} M, while the content of DNA or chromatin was varied by dilution of the initial sample in the reporter–buffer solution. DNA or chromatin, appropriately diluted in sodium phosphate buffer lacking reporter, was employed as a reference. Values utilized in calculations of the amounts of free and bound reporter were ϵ_{350} 1.66×10^4 M^{-1} cm^{-1} for the free reporter (Gabbay, 1969), ϵ_{345} 1.04×10^4 M^{-1} cm^{-1} for reporter bound to chromatin, and ϵ_{345} 0.79×10^4 M^{-1} cm^{-1} for reporter bound to DNA. The extinction of reporter bound to chromatin was determined from trough to peak ratios in gel filtration experiments, or by dissociation of the isolated reporter chromatin complex in 0.7 M NaCl (*vide infra*). The extinction of reporter bound to DNA was determined by measurements of absorbance of 2.5×10^{-5} M reporter in the presence of large excesses of DNA ($2–8 \times 10^{-3}$ M DNA-phosphate).

Results

The reporter molecule (I) has previously been investigated by Gabbay and his coworkers, in careful studies of the spectral, circular dichroic, and nuclear magnetic resonance characteristics of the complexes of this and related molecules with native and denatured DNA, RNA, and synthetic polynucleotides (Gabbay, 1968, 1969; Gabbay and Mitschele, 1969; Gabbay *et al.*, 1969; Passero *et al.*, 1970). This reporter

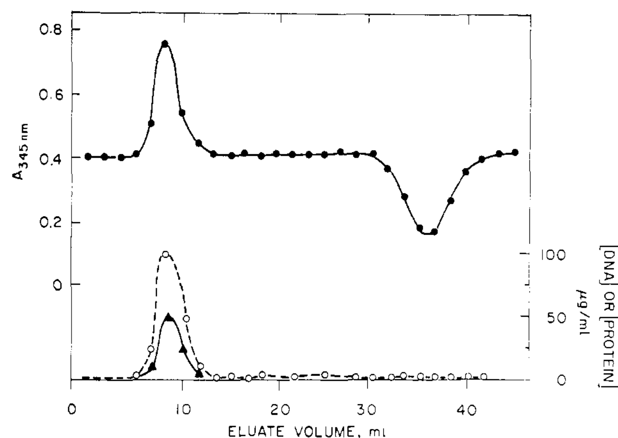
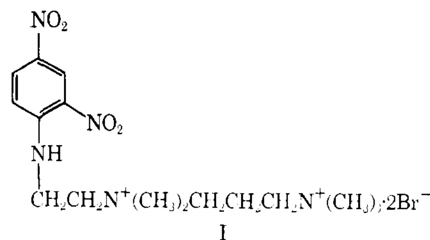


FIGURE 1: Gel filtration study of binding of the reporter molecule to chromatin. A 1.2 × 25 cm column of Bio-Gel P-60 equilibrated with 9×10^{-3} M sodium phosphate–2.5×10^{-5} M reporter, pH 7.3, 25° was employed. A 2-ml sample of chromatin, 5×10^{-4} M in DNA-phosphate, was applied to the column in the equilibrating buffer, and eluted with the same solvent. The presence of reporter is measured by absorbance at 345 nm (●—●). Protein contents of the various fractions were determined by the Lowry procedure (○---○). DNA content (▲—▲) was determined by absorbance at 260 nm.



has been thought to bind electrostatically to adjacent phosphate anionic charges on the backbone of DNA, with partial intercalation of the side chains of the aromatic substituent into the DNA base pairs (Gabbay, 1969; Passero *et al.*, 1970). In the current investigation, the interaction of the reporter molecule with chromatin has been demonstrated by three different approaches: equilibrium studies of binding by gel filtration, circular dichroism measurements on the bound reporter molecule, and spectrophotometric measurements of hypochromicity of the reporter consequent to its interaction with chromatin. The results of these three studies will be considered in turn.

The binding of the reporter molecule to chromatin is most clearly demonstrated by the results of a Hummel and Dreyer (1962) gel filtration experiment. In such an experiment, a column of Bio-Gel P-60 is equilibrated with a solution of the reporter. A band of chromatin is then passed through the column. A peak of reporter absorbance over the base line emerges with the large molecules, corresponding to the amount of reporter bound to the chromatin. A trough, also corresponding to the amount of bound reporter, emerges at the salt position. Such results are illustrated in Figure 1. Conservation

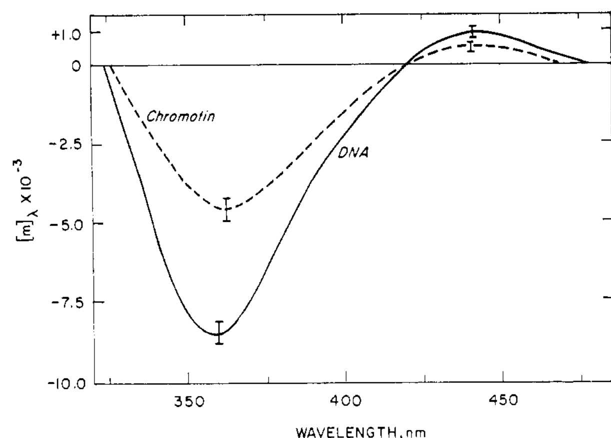


FIGURE 2: Circular dichroism spectra of the reporter interacting with DNA (——) or chromatin (- - -). Both sets of spectra were obtained with 1×10^{-4} M reporter in 9×10^{-3} M sodium phosphate buffer, pH 7.3 and 25°. The concentration of DNA-phosphate in DNA was 8×10^{-3} M, while that for chromatin was 2.7×10^{-3} M. Under these conditions the ratio of bound to free reporter molecule is 600 for DNA and 100 for chromatin. The vertical bars indicate the noise level of the recordings.

of mass requires that the amount of reporter in the peak and the trough be equal. Hence, if there is no alteration in the absorbance of the reporter when it binds, the integrated absorbance times volume areas will also be equal for the peak and trough. In the present case, the area of the trough is clearly larger than that of the peak (Figure 1), due to the hypochromism induced in the absorption of the reporter when it is bound to chromatin. The ratio of these two areas is a measure of the degree of hypochromism. Using this ratio and the known extinction coefficient of the free reporter, 1.66×10^4 M^{-1} cm^{-1} (Gabbay, 1969), the value of 1.04×10^4 M^{-1} cm^{-1} was calculated for the extinction of the reporter when bound to chromatin. A similar value, 1.01×10^4 M^{-1} cm^{-1} was obtained by dissociating the isolated reporter–chromatin complex with 0.7 M NaCl, and using the increase in absorbance under these conditions for calculation of the hypochromic effect.

Knowledge of the amount of reporter bound and the amount of chromatin applied to the column allows calculation of an individual point for a Scatchard (1949) plot. The points from three such gel filtration binding experiments are included in Figure 3, and agree well with the data obtained by another method of measurement of the stoichiometry and stability of the chromatin reporter complex (*vide infra*).

Such gel filtration experiments also allow evaluation of an important question in considerations of the binding of a cationic molecule, such as the reporter, to the chromatin complex, namely, whether the reporter binds to the nucleohistone complex *per se*, or alternatively, whether the reporter binds to DNA with the concomitant dissociation of the proteins of the chromatin. As shown in the lower portion of Figure 1, protein emerges only in the void volume, at the position expected for chromatin, and not near the salt volume, where free histones would have been eluted under these experimental conditions. Hence, it is apparent that the binding of the reporter to chromatin does not result in the dissociation of the nucleoprotein complex. The mass ratio of protein to DNA in the excluded peak is identical with that of the input sample of chromatin, 1.9 to 1.

The near-ultraviolet absorption spectrum of the reporter molecule, with a maximum at 350 nm and a shoulder at about 410 nm, is normally optically inactive. In the presence of DNA hypochromicity is induced in the absorption of the reporter, and the absorption bands of the reporter become optically active (Gabbay, 1969; see also Figure 2). Binding of the reporter to chromatin induces a circular dichroic spectrum qualitatively similar to that observed on its binding to DNA free in solution (Figure 2). For binding to DNA alone, a positive band is observed at 440 nm, with a molecular ellipticity of +1000, while a larger negative band is observed at 360 nm with a molecular ellipticity of −8500. These results are quite similar to those obtained by Gabbay (1969). When reporter is bound to chromatin, the positions and signs of the two circular dichroic bands are similar to those observed for free DNA (Figure 2). However, the magnitudes of the bands are reduced to about one-half of the values observed for the uncomplexed nucleic acid, *i.e.*, +500 and −4500, respectively.

The quantitative differences in ellipticities for the free DNA and chromatin–reporter complexes suggests that the degree of interaction between the transitions for reporter and nucleic acid bases is less in the case of chromatin. This difference is expected in view of the lessened hypochromicity of the reporter when bound to chromatin, as compared with when it is bound to DNA alone (*vide supra*). Irrespective of the quantitative differences in circular dichroism spectra between the reporter complexes with DNA and chromatin, the qualitative similarities of the two spectra suggest similar modes of interaction of the reporter with DNA in the two situations.

The marked hypochromism observed on the binding of the reporter to either DNA (Gabbay, 1969) or to chromatin (*vide supra*) made the use of the absorption properties of the reporter complexes the most expedient method for evaluation of stability and stoichiometry of the complexes formed between reporter and DNA or chromatin. In these studies, the nucleic acid content was varied, at a constant concentration of reporter molecule of 2.5×10^{-5} M in 9×10^{-3} M sodium phosphate buffer, pH 7.3 and 25°. The concentration of reporter bound to the nucleic acid, R_b, is determined from the observed absorbance, A, knowledge of the extinction coefficients of the free and bound reporter ϵ_f and ϵ_b, respectively, and the total reporter concentration, R_t.

$$R_b = \frac{(\epsilon_f R_t) - A}{\epsilon_f - \epsilon_b} \qquad (1)$$

The data obtained were analyzed by the Scatchard (1949) linear transformation of the general binding equation

$$\bar{n} = \bar{n}_{max} - \left(\frac{1}{K_a}\right)\left(\frac{\bar{n}}{R_f}\right) \qquad (2)$$

where $\bar{n}$ = the number of moles of reporter bound per mole of DNA-phosphate, $\bar{n}_{max}$ represents maximal binding, K_a is the association constant for the complex, and R_f is the concentration of unbound reporter. In a plot of $\bar{n}/R_f$ *vs.* $\bar{n}$, the intercept on the x axis is $\bar{n}_{max}$ while the intercept on the y axis is $(\bar{n}_{max})(K_a)$.

For both free DNA and DNA in chromatin apparently linear plots are obtained over the range of about 10–60% of maximal binding of the reporter (Figure 3). As previously noted, three points for chromatin–reporter interaction, obtained by equilibrium gel filtration studies, are included in this plot (triangles). They agree well with the data obtained from studies of the hypochromism, the latter experimental approach.

Least-squares linear fits to the data for both free DNA and chromatin indicate a maximal binding of 1 mole of reporter per 5.8 moles of DNA-phosphate (Figure 3). Previous investigations by other workers of the interaction of the reporter with DNA had not attempted to establish the maximal amount of binding attainable (Passero *et al.*, 1970). The present results suggest that the reporter binds to DNA in a ratio of one reporter molecule per six phosphates, or potentially, one reporter to three phosphates on a single chain, if the reporter indeed occupies one or the other groove of the DNA double helix.

The association constant for the reporter interacting with free DNA under these experimental conditions of pH and ionic strength is 7.5×10^4, somewhat higher than that previously obtained at a slightly higher ionic strength (Passero *et al.*, 1970), while that for the reporter–chromatin complex is 3.75×10^4, slightly less than the association constant for free DNA and the reporter molecule.

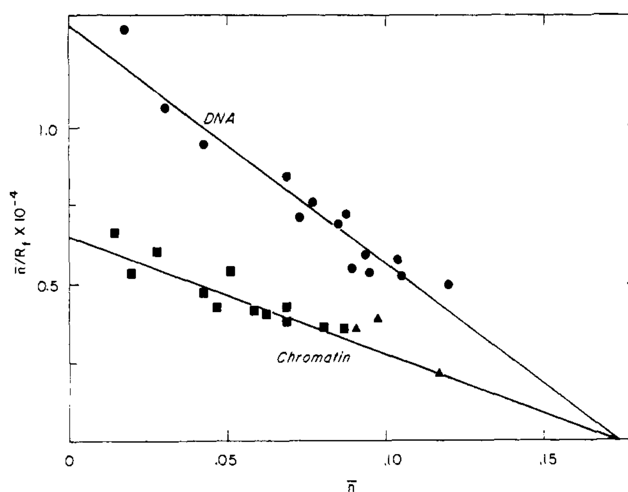


FIGURE 3: Reporter binding to DNA or chromatin. The Scatchard plots shown are derived from the study of the hypochromicity of reporter bound to DNA (●) or to chromatin (■). Data were obtained at 2.5×10^{-5} M reporter in 9×10^{-3} M sodium phosphate, pH 7.3 and 25°. Least-squares fits to the data are shown as the solid lines. Also included are three points (▲) obtained by gel filtration binding studies for chromatin.

Discussion

The general aim of our current investigations is to determine the structure of mammalian chromatin—that is, to understand the structure of the DNA in chromatin, the structure of proteins in chromatin, and particularly, the detailed contact interrelationships between these two major moieties of nucleohistone. Our initial studies in this line (Simpson and Sober, 1970), together with those of others, have demonstrated a significant alteration in the conformation of DNA when complexed in chromatin and major alterations in the conformation of the proteins of chromatin, when compared to their structure free in solution. Further, these investigations have suggested that the slightly lysine-rich histones play a major role in the maintenance of the altered conformation of the nucleic acid in chromatin (Zubay and Doty, 1959; Bradbury and Crane-Robinson, 1964; Ohba, 1966; Tuan and Bonner, 1969; Permogorov *et al.*, 1970; Shih and Fasman, 1970; Simpson and Sober, 1970). In the current investigation the goal has been to ascertain the location of the proteins of chromatin on the DNA of this complex species: the location as viewed in a section across the long axis of the chromatin. The most reasonable alternatives for this distribution are: (1) proteins located in both major and minor gooves of the DNA double helix, with or without bare regions lacking any protein cloak; (2) proteins located in the minor groove only; or (3) proteins located in the major groove only.

Of the various physicochemical approaches available for investigation of the current question, study of the interactions of a reporter molecule with chromatin has been chosen as the most expedient approach for the current study. The reporter employed has been exhaustively studied by Gabbay and coworkers in its interactions with native and denatured DNA, RNA, and synthetic polynucleotides. From their studies, it is apparent that this particular reporter molecule possesses characteristics quite appropriate for the current investigation. Thus, it appears to bind electrostatically to adjacent phosphate anions of the sugar phosphate backbone of the DNA helix, with intercalation providing only slight additional stabilization for its interaction with nucleic acids. Detailed circular dichroism studies of this and related reporters together with model building experiments, have suggested that the binding of the reporter to native DNA is in the minor groove of the double helix (Gabbay, 1969).

It was felt important in the current study to directly demonstrate the binding of the reporter to chromatin, to demonstrate that the interaction of the reporter with chromatin did not lead to dissociation of the components of nucleohistone, to evaluate the similarities of binding of the reporter to DNA in solution and to DNA in chromatin, and to establish the stoichiometry and stability of binding of the reporter both to DNA and to chromatin.

Gel filtration studies directly demonstrate the binding of the reporter to chromatin (Figure 1). Further, they allow determination of the hypochromicity attendant to binding of the reporter to the nucleohistone complex, and also demonstrate that the proteins of chromatin are not dissociated consequent to binding of the reporter molecule. Hence, the species studied in detail by measurements of hypochromism on binding of the reporter to chromatin is indeed a true complex of the small molecule with nondissociated nucleohistone.

The circular dichroism spectra of the reporter bound to DNA and to chromatin suggest that the mode of interaction of the small molecule with these two species of nucleic acid is similar. Both the band signs and the wavelength positions of the maxima are nearly identical in the two cases (Figure 2). For other reporters, *e.g.*, acridine orange, circular dichroism spectra have indicated different modes of interaction for the dye with chromatin and with free DNA (Simp-

son and Sober, 1970). In the current case, the similarities of spectra lend credence to the interpretation of binding studies as reflecting the same mode of interaction of the reporter with DNA in both free DNA and complexed nucleic acid in chromatin.

The decreased ellipticity observed for the reporter–chromatin complex, when compared to the reporter–DNA complex, is consistent with a less efficient coupling of the transition moments of the reporter and the nucleic acid bases. A lessened coupling would be expected if previously postulated models for the conformation of DNA in chromatin were to be correct, that is, a conformation in which the DNA base pairs are either partially unstacked, or tilted with respect to the major axis of the DNA double helix (Ohba, 1966; Tuan and Bonner, 1969; Bonner and Tuan, 1968; Permogorov *et al.*, 1970; Simpson and Sober, 1970).

The detailed stoichiometric investigations of the interaction of the reporter molecule with chromatin and DNA are of most particular interest with regards to the general question which led to these studies. If it is assumed that the reporter binds to adjacent phosphate anions in the minor groove of DNA, three different results would be expected in such binding studies with chromatin, corresponding to the three laternative distributions of proteins on the DNA helix listed previously. If protein cloaked the DNA totally, in both major and minor grooves, then no binding of the reporter to chromatin would be expected except for areas in which protein was absent. By other studies, the proportion of such areas would be expected to be of the order of 10–20% of the total DNA-phosphate content (Paul and Gilmour, 1966; Bonner *et al.*, 1968; Bekhor *et al.*, 1969). If the proteins of chromatin were localized solely in the minor groove of the DNA, no reporter binding would be expected. As the third alternative, if the proteins were confined to the major groove of the DNA helix, and a large portion of the phosphate negative charges were available, then the same number of binding sites for the reporter would be found in both chromatin and free DNA.

The experimental results obtained (Figure 3) are consistent only with the third of these three alternatives. The number of binding sites for reporter for both DNA and chromatin is identical, indicating no effect of the presence of histones and nonhistone proteins on the availability of binding sites for the small molecule. If indeed, the reporter occupies the minor groove when interacting with DNA, such a result is possible only when the proteins of chromatin do not occupy this area. It thus seems most likely that the proteins of chromatin are confined to the major groove of the DNA double helix. These results do not, of course, preclude the presence in chromatin of some of the protein in yet other arrays, *e.g.*, cross-linking the strands of DNA to form condensed structures.

The conclusion that the minor groove of chromatin is available for interaction with the reporter is strengthened by the high proportion of the total potentially available sites which are bound by the reporter at saturation. Maximal binding occurs at a ratio of one reporter molecule per six phosphate anions of the nucleic acid. If the reporter lies in the minor groove, steric considerations would seem to preclude the binding of reporter molecules to diametrically opposed pairs of phosphates on the two chains, and hence, these results might imply binding of one reporter to three phosphates along one chain of the DNA double-helical structure. The dicationic structure of the reporter binding group requires binding to two adjacent phosphate anions along one chain, and it seems not unlikely that the bulk of the aromatic substituent of the reporter might block electrostatic interaction of another reporter molecule with yet a third phosphate for each molecule bound. If so, these results suggest that a ratio of one reporter to six phosphates, that found experimentally, might represent truly maximal saturation of all possible binding sites on DNA.

The availability of a large fraction of the negative charges of the DNA of chromatin for interaction with the reporter (or other molecules), and the putative demonstration that the minor groove of the double-helical structure of DNA in chromatin is not filled with proteins, have implications for the mechanisms of restriction of transcription in mammalian cells. This subject will be considered in furture communications.

Acknowledgments

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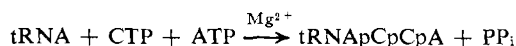
Purification and Characterization of Yeast Nucleotidyl Transferase and Investigation of Enzyme–Transfer Ribonucleic Acid Complex Formation*

Robert W. Morris† and Edward Herbert

ABSTRACT: Nucleotidyl transferase has been purified more than 1000-fold from yeast. Autolysis of yeast is followed by ammonium sulfate precipitation, gel filtration, DEAE-cellulose chromatography and, finally, hydroxylapatite chromatography. These last three steps are performed in 40% (v/v) glycerol. After the last step, the enzyme is essentially free of ribonucleases, phosphomonoesterases, phosphodiesterases, pyrophosphatases, and aminoacyl-tRNA synthetases.

The optimum concentrations for the components of the reaction are: ATP, 2 mM; CTP, 150 μM; and Mg^{2+}, 10 mM. The optimum pH is 9.5. When the hydroxylapatite fraction is sedimented in a sucrose density gradient, the enzymatic activity is found as a single symmetric peak sedimenting slightly more slowly than homoglobin (4.3 S). However, in the presence of tRNA, the enzymatic activity sediments 50% faster ($\sim$6 S). Disc gel electrophoresis patterns of the hydroxylapatite fraction show two bands of approximately equal density. The faster moving of these bands predominates in disc gel patterns of fractions from the enzyme region ($\sim$6S material) of a sucrose density gradient containing enzyme and tRNA, indicating that the enzyme is in the faster moving gel band. Two kinds of evidence are presented to show that a stable complex is formed between the transferase and tRNA. The first is sedimentation patterns in sucrose density gradients like those described above, and the second is enzyme-dependent binding of radioactive tRNA to nitrocellulose filters. Enzyme-dependent binding occurs in the presence or absence of ATP, CTP, or Mg^{2+} and shows little dependence on pH in the range 6.5–7.5. The enzyme binds very tightly to any tRNA that is missing part of the terminal pCpCpA triplet, even if a 3′-phosphate group is present. If, however, the terminal triplet is complete, the complex is very much weaker. The transferase cannot bind small fragments resulting from T_1 RNase digestion, nor can it bind polyuridylic or polycytidylic acids. While the last two polynucleotides do not bind, they do inhibit the enzyme-dependent binding of tRNA.

Nucleotidyl transferase catalyzes the addition of AMP and CMP to tRNA, forming the terminal pCpCpA triplet (Starr and Goldthwait, 1963; Preiss *et al.*, 1961; Hecht *et al.*, 1958; Herbert, 1959). This reaction has been formulated as follows



$$\text{tRNA} + \text{CTP} + \text{ATP} \xrightarrow{Mg^{2+}} \text{tRNApCpCpA} + PP_i$$

In order to study this reaction, the nucleotidyl transferase from yeast has been purified free of enzymes that degrade or modify the substrates or products of this reaction.

Two major difficulties in the previous studies of this reaction have been that the tRNA used as the substrate was inhomogeneous and of unknown and presumably inhomogeneous end-group composition. That is, bulk tRNA is a mixture of many amino acid acceptor species with at least three different end groups: tRNApXpC, tRNApXpCpC, and tRNApXpCpCpA,[1] where X is the undefined fourth nucleotide.

In the present study, advantage was taken of recent advances in tRNA purification by using tRNAs enriched for a single amino acid acceptor tRNA. The second difficulty mentioned above was overcome by the use of the periodate–amine degradation technique developed by Khym and Uziel (1968) which permits stepwise removal of nucleotides from the pCpCpA end of tRNA.

The use of yeast as the source of enzyme and tRNA pro-

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[1] Abbreviations used are: tRNApYpXpCpCpA is tRNA with the complete pCpCpA triplet and X and Y are the fourth and fifth nucleotides, respectively.