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# Measurement of DNA–Protein Equilibria Using Gel Chromatography: Application to the *Hin*fI Restriction Endonuclease†

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ABSTRACT: A method is described for measuring equilibrium constants of DNA–protein interactions using gel chromatography. This technique has been used to study the sequence-specific interaction of the *Hin*fI restriction endonuclease with DNA. *Hin*fI has a monomeric molecular weight of 31 000 and exists as a dimer in its active form. The protein binds to supercoiled DNA molecules containing its recognition site with an apparent free energy of −13.9 kcal/mol of sites. This interaction is highly salt sensitive and causes a release of 3.4 ion pairs. The affinity of the nuclease for its recognition site is largely independent of both pH (6.5–8.5) and temperature (7–35 °C) and was not affected by variations in the degenerate middle position of the site. Linear DNA fragments containing the *Hin*fI recognition site were bound as tightly as supercoiled molecules. Binding to nonspecific DNA sites or to methylated DNA sites was approximately 6 orders of magnitude weaker. In general, enzyme activity and binding affinity paralleled each other.

Type II restriction endonucleases provide good model systems for the study of sequence-specific recognition of DNA by proteins. One of the first steps in examining such interactions is the accurate determination of the equilibrium binding constants with specific and nonspecific DNA sites.

The most widely used method employs nitrocellulose filter binding (Riggs et al., 1970). This method is based on the observation that DNA alone will pass through a nitrocellulose filter while protein–DNA complexes are retained. A variety of other techniques have been described in addition to filter binding. Ackers et al. (1983) have developed methods for the

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quantitative analysis of DNA footprinting data and have applied them in determining binding constants for the λ repressor to its operator sites. Affinity chromatography has been used to measure protein binding to nonspecific DNA (de Haseth et al., 1977). Gel electrophoresis has been successful in studying DNA–protein interactions (Fried & Crothers, 1981; Garner & Revzin, 1981). Other methods employ an analysis of the nonequilibrium transport properties of the interacting species during centrifugation (Saxe & Revzin, 1979; Draper & von Hippel, 1979; Revzin & Woychik, 1981).

The gel chromatography procedure described in this paper is also based on transport and is used to measure the equilibrium binding constant of the *Hinf*I restriction endonuclease to specific and nonspecific DNA sites. The *Hinf*I enzyme was chromatographed on Sephadex G-200 in the absence and presence of DNA in the elution buffer. The equilibrium constant could then be calculated from the known concentration of sites in the DNA and the ratio of elution volumes. We believe this method is simple enough to be useful particularly in cases where the filter binding method is not applicable.

*Hinf*I is an interesting enzyme for study. It recognizes the degenerate sequence 5′-G-A-N-T-C (where N is any base) and cleaves between the guanine and adenine residues on each strand of its duplex site (Roberts, 1983). Hence, one important question concerns the possible influence of the choice of the degenerate base on the binding constant. In addition, several other restriction enzymes recognize the same sequence. Among these is *Hha*II, the gene for which has been previously cloned (Mann et al., 1978) and sequenced (Schoner et al., 1983). It is of interest to compare these enzymes to determine whether they recognize the sequence in a similar fashion.

## Materials and Methods

*DNAs.* Plasmid pBR322 (Bolivar et al., 1977) and pDI10 (Mann et al., 1978) DNAs were purified from *Escherichia coli* HB101 cells by the rapid alkaline extraction procedure of Birnboim & Doly (1979) and equilibrium centrifugation in CsCl–ethidium bromide gradients (Clewell & Helinski, 1970). Plasmid pDI10 carries the *Hha*II restriction and modification genes and is methylated at the *Hha*II (*Hin*fI) sites. DNA fragments were prepared by *Taq*I or *Hinf*I restriction enzyme digestion and purified by gel electrophoresis and electroelution (Smith, 1980). DNA concentrations were measured by the absorbance at 260 nm (1 μg = 0.02 absorbance unit).

*Proteins.* The *Hinf*I endonuclease was purified by Michael Nelson (New England Biolabs, Beverly, MA). The protein purity (>95%) and concentration were estimated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli, 1970) followed by silver staining (Morrisey, 1981). Protein standards used for electrophoresis and chromatography were cytochrome *c*, soybean trypsin inhibitor (SBTI), chymotrypsinogen A, aldolase, ovalbumin, catalase, and bovine serum albumin (BSA) and were obtained from Sigma Chemical Corp.

*Hinf*I *Endonuclease Assay.* Enzyme assays were carried out in reaction mixtures (50 μL) containing 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, 100 μg/mL bovine serum albumin, 100 μg/mL pBR322 DNA, and *Hinf*I endonuclease (0.01–1.0 unit as adjusted to yield partial digestion of the plasmid DNA). Incubation was at 37 °C for 60 min. The extent of the reaction was determined by electrophoresis on 1% agarose gels (Sharp et al., 1973) followed by staining with ethidium bromide. The peak of activity was located by either densitometry or direct visualization of the product bands.

*Kinetics.* Reaction mixtures were the same as those employed for binding (see below) except that 10 mM $MgCl_2$ was included. The DNA used was supercoiled pBR322 which contains 10 sites for *Hinf*I. Samples were removed at various time intervals, and the products were separated by agarose gel electrophoresis. The gels were stained with 0.5 μg/mL ethidium bromide (Sharp et al., 1973) and photographed. The intensity of the initially produced linear product band was quantitated by densitometry and the initial rate calculated.

*Chromatography and Standard Hinf*I *Binding Conditions.* For gel chromatography, a water-jacketed 1 × 30 cm glass column, fitted with a polyethylene disk, was clamped vertically and attached to a circulating thermostated water bath. Sephadex G-200 (Pharmacia), suspended in water, was packed under a pressure of 20 cm and allowed to equilibrate at the proper temperature. Flow of buffer through the column was controlled at 5–6 mL/h by a peristaltic pump. *Hinf*I binding reaction mixtures (100 μL) contained 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 100 μg/mL bovine serum albumin (>99% purity, Calbiochem), an appropriate concentration of DNA, and *Hinf*I endonuclease. The reaction mixture was incubated at the column temperature for 15 min. It was then loaded and chromatographed on a column which had been preequilibrated with the reaction buffer containing DNA but no enzyme. All experiments were carried out in the absence of $MgCl_2$ to prevent cleavage of the DNA. Chromatography was generally carried out at 21 °C, 0.35-mL fractions were collected, and the eluted activity was assayed under the conditions described above.

*Theory of the Binding Method.* Even relatively small (≤200 base pairs) DNA molecules are completely excluded from Sephadex G-200 beads. Hence, a protein bound to this DNA will also be totally excluded. Unbound protein, on the other hand, will be in the included volume if its molecular weight is within the fractionation range of the gel. To exploit these properties for the measurement of equilibrium binding constants, one passes a small zone of protein through a column equilibrated with a particular concentration of DNA. Assuming a rapid equilibrium with the DNA in the column, the protein will partition itself partly in the stationary phase when unbound and entirely in the void volume when bound to DNA. When the protein is totally unbound (the DNA concentration significantly below the binding constant), it will elute according to its molecular weight. When fully bound, it will elute along with the DNA in the void volume. When partially bound, the elution volume will fall between the two extremes in proportion to the time spent bound to DNA on the column. By treating the protein as the ligand, one can calculate an association constant, $K_a$, for the interaction by

$$K_a[D] = (V_p - V_e)/(V_e - V_d)$$

where [D] is the DNA site concentration equilibrated within the column, $V_p$ is the elution volume of the protein in the absence of DNA, $V_d$ is the elution volume of the DNA by itself (the void volume for large DNAs), and $V_e$ is the elution volume of the protein in the presence of DNA. It is assumed that the free DNA concentration is approximately equal to the total DNA concentration. Sephadex G-200 was chosen as the column resin to maximize separation between free protein and DNA. Analysis of the binding isotherms was carried out by a nonlinear least-squares fitting program on a Hewlett Packard 1000 minicomputer as described by Turner et al. (1981).

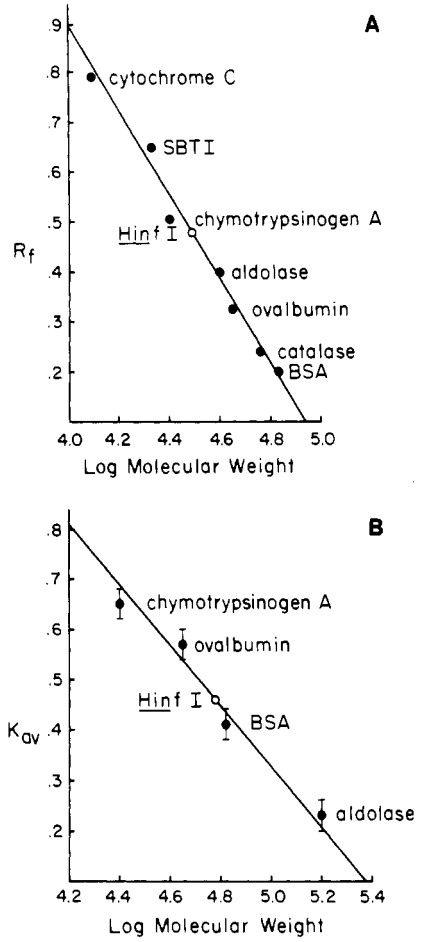


FIGURE 1: Determination of the subunit and native molecular weights of the *Hinf*I restriction endonuclease. Panel A is a plot of the relative mobilities of protein standards on a 12% sodium dodecyl sulfate–polyacrylamide gel as a function of subunit molecular weight. The molecular weight of *Hinf*I was found by interpolation. Panel B shows the plot used to determine the native molecular weight of *Hinf*I. Data were obtained by finding the elution volume of *Hinf*I endonuclease activity and of protein standards (assayed by absorbance at 280 nm) on a Sephadex G-200 column. Error bars indicate fraction size.

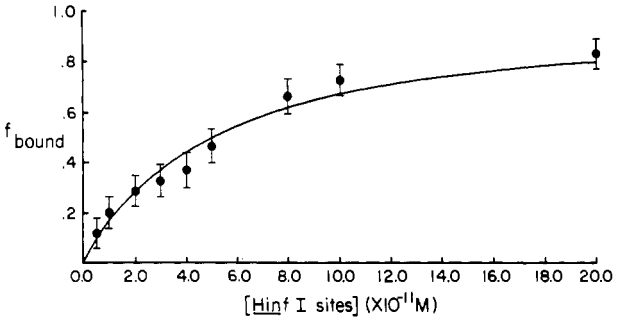


FIGURE 2: Binding isotherm for the interaction of *Hinf*I restriction endonuclease with pBR322 supercoiled DNA. Each point represents the fraction of protein bound to DNA at a particular DNA concentration within the column, and the error bars indicate the size of the fraction collected. DNA concentration is expressed as the concentration of *Hinf*I sites (10 per molecule).

## RESULTS

*Properties of the HinfI Restriction Endonuclease.* The subunit molecular weight of the enzyme was found to be 31 000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 1A). The native molecular weight was estimated by gel chromatography on a Sephadex G-200 column to be about 60 000 (Figure 1B). This suggests that the active enzyme exists as a dimer.

Table I: Binding to Different Substrates

| substrate[a] | $K_{obsd}$ ($M^{-1}$)[b] |
|---|---|
| pBR322 supercoils | $(1.98 \pm 0.36) \times 10^{10}$ |
| pBR322 *Taq*I fragments | $(3.88 \pm 1.45) \times 10^{10}$ |
| pBR322 *Hinf*I fragments | $(5.20 \pm 0.48) \times 10^{4}$ |
| pDI10 supercoils | $<1.43 \times 10^{5}$[c] |

[a] Substrates were prepared as described under Materials and Methods. [b] Measurements were in standard buffer (described under Materials and Methods) at 21 °C ± SD. [c] This value is an upper limit due to the high concentrations of substrate required to observe binding.

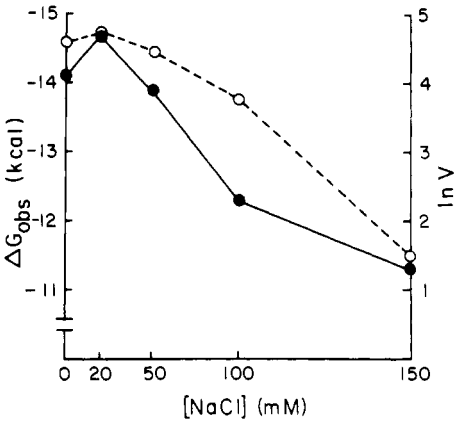


FIGURE 3: Salt concentration dependence of *Hinf*I endonuclease binding and cleavage rates. The solid line represents the observed free energies of binding to supercoiled pBR322 DNA calculated from the association constants as a function of NaCl concentration. A minimum of four DNA concentrations was used to determine each equilibrium constant. The broken line shows the initial velocity of the cleavage reaction (in the presence of $MgCl_2$) plotted as the natural log of the velocity for comparison with the free energies.

*Binding of HinfI to DNA.* The binding isotherm for *Hinf*I binding to pBR322 supercoiled DNA under standard conditions is shown in Figure 2. An apparent association constant of $(1.98 \pm 0.36) \times 10^{10}$ $M^{-1}$ and a free energy of −13.9 kcal/mol were calculated.

To determine the stoichiometry of *Hinf*I binding to DNA sites, several independent chromatographs were carried out under standard conditions with a fixed pBR322 DNA concentration and with increasing concentrations of *Hinf*I endonuclease over a 15-fold range. Saturation was achieved at an apparent stoichiometry of 0.87 mol of dimer per mole of *Hinf*I sites.

Binding of *Hinf*I to several other substrates was also measured under standard conditions (Table I). The affinity of the nuclease for a mixture of linear fragments of pBR322, ranging in size from 141 to 1444 base pairs from a *Taq*I digestion, is similar to that for supercoiled DNA. However, pBR322 DNA that had been extensively cleaved by *Hinf*I before measurement of the binding (nonspecific DNA) was bound almost 6 orders of magnitude more weakly. Here, we are assuming that all the specific sites were destroyed and that the affinity for the cleaved termini is low. All of the digest fragments were sufficiently large to be completely excluded from the gel. In addition, *Hinf*I binding to plasmid pDI10, methylated at the adenine position of the *Hinf*I sites, was reduced by at least 5 orders of magnitude. In calculating the association constants to the latter two substrates, it was assumed that each base pair could initiate a potential nonspecific binding site.

*Salt Dependence.* The equilibrium constant for the binding of *Hinf*I to plasmid pBR322 DNA was determined as a function of NaCl concentration. Figure 3 shows that binding is strongly dependent on the salt concentration. In addition,

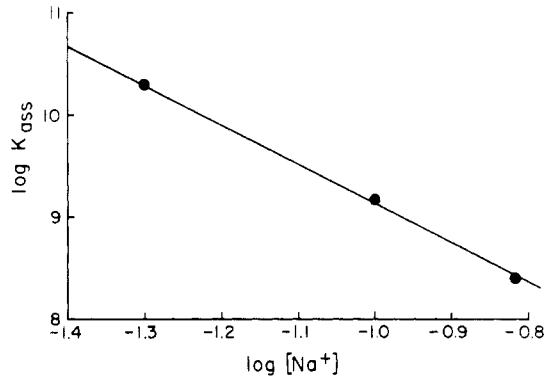


FIGURE 4: Determination of the number of ion pairs released upon *Hinf*I endonuclease binding to supercoiled pBR322 DNA. The slope of the plot corresponds to the number of ion pairs released, $m'$, as calculated by the theory of Record et al. (1976). Data are shown from 50 to 150 mM NaCl, where the plot is linear.

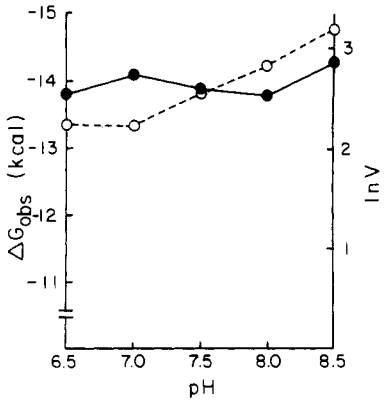


FIGURE 5: pH dependence of *Hinf*I endonuclease binding and cleavage rates. The solid line represents the apparent free energies of binding to supercoiled pBR322 DNA calculated from the association constants at various pHs. At least four DNA concentrations were used to determine each equilibrium constant. The broken line presents the natural log of the initial velocity of the cleavage reaction in the presence of $MgCl_2$.

the dependence on salt of the cleavage reaction, plotted as the logarithm of the initial velocity, is shown to parallel that for binding (Figure 3). From the theory of Record et al. (1976, 1977) and assuming no anion effects, one can calculate the number of ion pairs released upon binding from the salt dependence of the interaction using the relationship

$$-\text{d log } K_{obsd}/\text{d log } [M^+] = m'\psi$$

where $[M^+]$ is the monovalent counterion concentration, $\psi$ is the fraction of counterion bound per phosphate (assumed to be 0.88 for double-stranded DNA; Record et al., 1976), and $m'$ is the number of ion pairs formed. From the slope of the plot shown in Figure 4, a value for $m'$ of 3.4 ion pairs formed was determined.

*pH and Temperature Dependence.* The effect of pH both on binding and on the cleavage rate is shown in Figure 5. There is very little change in the equilibrium constant over this range of pH (6.5–8.5) and possibly a minor enhancement of the reaction rate at the higher pHs. The temperature dependence of the interaction between the *Hinf*I endonuclease and pBR322 DNA was studied by varying the temperature in the column water jacket from 7 to 35 °C. The results are plotted in Figure 6. The free energy of the interaction increases slightly at higher temperatures.

*Effect of the Degenerate Middle Position.* Since the recognition site for *Hinf*I is degenerate at the middle position, it was of interest to study the effect of base pair variations at

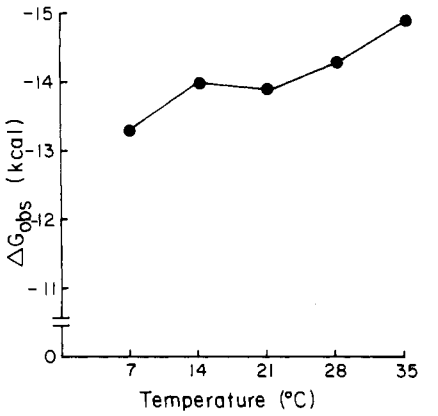


FIGURE 6: Temperature dependence of *Hinf*I endonuclease binding. Each point corresponds to the free energy of interaction between *Hinf*I and pBR322 supercoiled DNA. These were calculated from the observed equilibrium constants over the range of temperatures indicated. At least four DNA concentrations were used to determine each point.

Table II: Individual Site Binding

| site | fragment size (base pairs) | $K_{obsd}$ ($M^-$)[a] | initial velocity (fmol/min)[b] |
|---|---|---|---|
| 5′GATTC | 335 | (2.43 ± 0.43) × $10^{10}$ | 14 |
| 5′GAATC | 226 | (3.92 ± 0.54) × $10^{10}$ | 19 |
| 5′GACTA | 281 | (1.50 ± 0.18) × $10^{10}$ | 10 |
| 5′GAGTC | 257 | (4.33 ± 1.73) × $10^{10}$ | 13 |

[a] Measurements in standard buffer at 21 °C ± SD. [b] Measured under standard conditions at 21 °C in the presence of $MgCl_2$.

this position on binding and cleavage. Four fragments were isolated from pBR322, each containing a single site. Since the sequence is a palindrome, there are only two possible kinds of sites, one containing a GC base pair in the middle and the other an AT base pair. Binding measurements were made for each fragment and cleavage rates measured by densitometry of product bands on polyacrylamide gels. These data are presented in Table II. There appears to be little effect, caused by varying the middle base pair, on either the association constant or the cleavage rate.

## DISCUSSION

The method described here is well suited for the study of DNA–protein interactions. It is possible to measure a wide range of equilibrium constants while using only catalytic amounts of protein. The technique is simple although more time consuming than the widely used nitrocellulose filter binding assay (Riggs et al., 1970). However, some proteins may not bind to nitrocellulose. For example, we were unable to find conditions (by varying salt, buffer, solvent, or flow rate) under which the *Hinf*I restriction endonuclease or the protein complexed with DNA could be retained on nitrocellulose filters. The accuracy of our gel chromatography method depends on the separation between the protein and DNA on the column, which is usually quite large. In addition, within certain limitations, it may be possible to measure association constants with an impure protein preparation since one is assaying for activity. An impure preparation (<5%) of *Hinf*I, for example, yielded an association constant identical with that of the pure protein.

There are, however, a number of disadvantages to using this method. As noted above, measurements take a relatively long period of time although it may be possible to find high-pressure liquid chromatography (HPLC) resins to speed up the process. Some proteins interact with the Sephadex resin, making it impossible to measure their equilibria. The protein must be

stable over the period of time necessary to run the column. Multimeric proteins may dissociate as the protein is diluted during the course of the column run. The method is most suited to enzymes, rather than binding proteins, for the following reason. Since a very small concentration of protein is used in the column, it is difficult to measure the peak protein fraction unless one has an assay independent of the binding itself. For the same reason, it is not possible to use this method for enzymes with low specific activities.

The present work has focused on the *Hin*fI restriction endonuclease interactions with DNA. This enzyme has a monomeric molecular weight of 31 000. Its native molecular weight was determined by using the same conditions and column as the binding experiments and was found to have an apparent molecular weight of 60 000. Therefore, it is probable that the protein binds to DNA as a dimer. Consistent with this finding is the observed stoichiometry of binding of 0.87 mol of dimer per mole of *Hin*fI site in titration experiments. The functional form of the *Eco*RI nuclease also appears to be dimeric (Terry et al., 1983). As is also true for *Eco*RI (Rosenberg et al., 1981), *Hin*fI was found to bind to DNA in the absence of $Mg^{2+}$ although, unlike *Eco*RI, it is unable to bind to nitrocellulose filters under a variety of reaction conditions.

The interaction of *Hin*fI to specific and nonspecific DNAs has been examined. The enzyme binds to its recognition site, 10 of which are present on pBR322 supercoiled DNA, about 6 orders of magnitude more strongly than to an average nonspecific DNA site. The affinity for DNA methylated at its cleavage site is reduced to levels near that of nonspecific DNA. These observations are very similar to those found for the *Eco*RI restriction enzyme by Terry et al. (1983). They found that *Eco*RI binding to a nonspecific DNA site is about 5 orders of magnitude weaker than to its specific site and is reduced by at least 3 orders of magnitude to its methylated site. In addition, *Hin*fI binding occurred equally well to supercoiled DNA and linear fragments as does *Eco*RI (Terry et al., 1983). Although other studies of *Eco*RI binding to both specific and nonspecific DNAs have been carried out (Woodhead & Malcolm, 1980; Goeppelt et al., 1980; Jack et al., 1981; Rosenberg et al., 1981), they are difficult to compare due to the widely varying conditions used.

The association constant for the interaction of the *Hin*fI restriction endonuclease and pBR322 DNA is dependent on the salt concentration. Analysis of these data according to the polyelectrolyte theory of Record et al. (1976, 1977) shows that 3.4 ion pairs are released upon nuclease binding to its recognition site. This salt dependence is not quite as dramatic as that seen for *Eco*RI which releases 8.1 ion pairs (Terry et al., 1983); however, the *Hin*fI site is slightly smaller. In the case of *Eco*RI, the number of ion pairs released agreed quite well with the number of phosphate contacts predicted from the alkylation studies (Terry et al., 1983). By analogy, it would thus appear that *Hin*fI does not have as many contacts with the DNA backbone, possibly only three or four.

There is no change in affinity of *Hin*fI for its site over the range of pHs from 6.5 to 8.5. The dependencies of the initial cleavage reaction velocity on salt and pH display behavior very similar to that observed for binding. This suggests that the binding event may be the limiting step for catalysis. However, the effect of $MgCl_2$, present only for the cleavage reactions, is unknown. We assume that specific binding is the same with and without this ligand. Temperature had only a slight effect on the observed equilibrium constant, again being similar to that seen for *Eco*RI (Terry et al., 1983).

It has been observed that some restriction enzymes can cleave their canonical sites with different efficiencies (Thomas & Davis, 1975; Forsblom et al., 1976; Rubin & Modrich, 1978; Armstrong & Bauer, 1982; I. Schildkraut, personal communication) and that *Eco*RI binds to sites on λ DNA with different affinities (Halford et al., 1980). This effect has been attributed to the flanking sequences surrounding each site. To address the question of the effect of external sequences around the *Hin*fI site, and also to examine the effect of the internal degenerate nucleotide position, we measured the association constant and cleavage rate for four different DNA fragments each containing one *Hin*fI site. Neither the equilibrium constant nor the cleavage rate varied significantly among any of the fragments.

It will be most informative to extend this study to several other proteins which share a common recognition site with *Hin*fI. Preliminary results with the purified *Hha*II endonuclease show many differences between the two proteins (S. Kelly and R. Kaddurah, personal communication). For example, there is little or no homology between the *Hha*II gene and the *Haemophilus influenza* genome, suggesting little protein homology. The *Hha*II nuclease forms a dimer of 50 000 daltons, compared to 60 000 daltons for *Hin*fI, and the specific activity of *Hin*fI appears to be at least 10-fold higher than that of *Hha*II. More detailed comparisons are presently under way.

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**Registry No.** *Hin*fI, 81295-23-0.

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# Influence of Monovalent Cation Transport on Anabolism of Glycosphingolipids in Cultured Human Fibroblasts†

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ABSTRACT: We have reported [Saito, M., Saito, M., & Rosenberg, A. (1984) *Biochemistry 23*, 1043–1046] that the monovalent cationic ionophore monensin reduced the incorporation of labeled galactose into oligosaccharidyl glycosphingolipids (globotriaosylceramide, globotetraosylceramide, and gangliosides) and induced a cellular accumulation of glucosyl- and lactosylceramide in cultured diploid human fibroblasts. We have undertaken further studies on the effects of monensin and made comparison with the effects of related monovalent cation transporters on plasma membrane glycosphingolipid anabolism in human fibroblasts. Our results demonstrate that ionic flux can markedly influence glycosphingolipid synthesis, and they indicate that, like glycoprotein, the sites of glycosylation of the initial, precursor glycosphingolipids are different from the sites of higher glycosylation. At a concentration of $10^{-7}$ M, monensin induced the maximum inhibition of incorporation of labeled galactose into polyglycosyl sphingolipids: globotriaosylceramide, globotetraosylceramide, and gangliosides; increased incorporation of labeled galactose into glucosyl- and lactosylceramide was clearly evident, and their content rose measurably in the cell at concentrations of monensin as low as $10^{-8}$ M. These effects of monensin were reversible. Incorporation of labeled galactose into higher glycosylated neutral glycosphingolipids and gangliosides slowly resumed, and the accumulated glycosylceramide diminished after removal of monensin from the culture medium. Ouabain (plasma membrane $Na^+,K^+$-ATPase inhibitor) and A23187 ($Ca^{2+}$ ionophore) also caused a rapid increase in incorporation of labeled hexose into glucosylceramide and decreased its incorporation into higher neutral glycosphingolipids and into gangliosides. The effects of the $K^+$ ionophore valinomycin and the lysosome-disrupting cation $NH_4^+$ were different from those of ouabain and monensin and showed a general enhancement of incorporation of labeled galactose into all glycosphingolipids and no accumulation. These findings suggest that manipulation of ionic flux may variably regulate anabolism and, consequently, the composition of plasma membrane glycosphingolipids.

Monensin, a monovalent cationic ionophore, has been shown to arrest intracellular transport of newly synthesized proteoglycans, secretory proteins, and plasma membrane glycoproteins within the Golgi complex [for a review, see Tartakoff (1983)]. Therefore, monensin inhibits some of the posttranslational modifications of proteins (Tartakoff, 1979; Tartakoff et al., 1981; Tajiri et al., 1980; Nishimoto et al., 1982; Townsend & Benjamins, 1983; Crine & Defour, 1982) and the synthesis of mucopolysaccharides (Goldberg & Toole, 1983), which are believed to occur in the Golgi apparatus.

Elongation of sugar chains in glycosphingolipids through stepwise addition of monosaccharide units to the nonreducing end of the lengthening oligosaccharide chain by a "multiglycosyltransferase complex" (Roseman, 1970; Li & Li, 1982; Basu & Basu, 1982; Kishimoto, 1982; Ledeen, 1983) has been shown to occur mainly in the Golgi apparatus (Keenan et al., 1974; Wilkinson et al., 1976; Richardson et al., 1977; Pacuszka et al., 1978). We have demonstrated that manipulation of cationic flux by monensin, the monovalent cationophore, influences glycosphingolipid synthesis in human fibroblasts, and we have obtained suggestive evidence that the sites of glycosylation of glucosyl- and lactosylceramide are different from those of higher glycosylated glycosphingolipids (Saito et al., 1984), which is not consistent with the multiglycosyltransferase concept. In this study, we have further analyzed the monensin

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