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A 'one-pot' assay for the accessibility of DNA in a nucleosome core particle

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ABSTRACT

The accessibility of nucleosomal DNA to transcription factors and other sequence-specific DNA binding proteins is of importance in the consideration of mechanisms of transcriptional control. Here, we report a simple novel assay which determines this accessibility at eight different rotationally equivalent sites on nucleosomal DNA and shows that linker histones and the chromosomal HMGB proteins, HMG-D and HMG-Z, have opposite effects on the accessibility of nucleosomal DNA. We compare this assay to previously described methods.

INTRODUCTION

To address the question of how transcription factors might access DNA target sites bound in a nucleosome core particle, Widom and co-workers (1–3) developed an assay for DNA accessibility by introducing a number of different restriction sites into a high-affinity nucleosome binding site. The designed sequence, 601.2, was then reconstituted into uniquely positioned nucleosomes and the rates of digestion by the different restriction endonucleases determined. The resulting data showed that the rate of digestion decreased progressively from the outermost to the most central site. On this basis, Widom (4) proposed that the accessibility of nucleosomal DNA to other DNA binding was dependent on transient unwrapping from one or other end of the wrapped DNA.

The assay, as originally designed, although in our hands entirely reproducible (5), contains several potentially complicating variables. The rotational position of the restriction sites with respect to the surface of the histone octamer is one such variable as also are the mechanisms and kinetic constants of the restriction enzymes used. We have sought to simplify these variables by creating a set of nucleosome binding sites, based on the 601.2 sequence, that differ only in the position of a single restriction site. In all the constructs, the rotational orientation of this restriction site is the same. With this system, we can assay in a single reaction the accessibility of the wrapped DNA at many different sites. In addition, we found it necessary to modify the published working equations for calculating the individual rate constants. Our data are not wholly consistent with the progressive transient unwrapping of nucleosomal DNA as a mechanism for generating accessibility to restriction enzymes (4,6). Instead, we suggest that such a mechanism may, on this nucleosome, be restricted primarily to the outermost contacts with histones H2A and H2B, while at the contacts with histones H3 and H4 accessibility may also be generated by transient local breakage of histone–DNA contacts.

THEORY

Consider an equilibrium of two nucleosome conformations (N, n), where only n is the conformation of nucleosomal DNA is susceptible to cleavage by a restriction enzyme

$$N \underset{k_2}{\overset{k_1}{\leftrightarrow}} : n + E \underset{k_4}{\overset{k_3}{\leftrightarrow}} En \xrightarrow{k_p} E + P \qquad \textbf{Scheme a}$$

The event to the right of the dotted line follows the classical Michaelis–Menten relationship:

$$\frac{dn}{dt} = v = \frac{[n] \cdot V_{max}}{K_m + [n]} \qquad \textbf{1}$$

If $[n] \ll K_m$, Equation 1 reduces to

$$\frac{dn}{dt} = \frac{V_{max}}{K_m} \cdot [n]$$

Define $K = V_{max}/K_m$, $dn/dt = K \cdot [n]$.

Schemea simplifies to

$$N \underset{k_2}{\overset{k_1}{\leftrightarrow}} n \overset{K}{\leftrightarrow} P \qquad \textbf{Scheme b}$$

The rate of reduction of N, n can be written as:

$$-\frac{dn}{dt} = (k_2 + K)n - k_1 N$$

$$-\frac{dN}{dt} = k_1 N - k_2 n$$

Solving the pair:

$$n = n_0 \cdot e^{bt} - [(b + k_2 + K)n_0 - k_1 N_0](b - c)^{-1}\left(e^{bt} - e^{ct}\right), \qquad \textbf{2}$$

$$N = [k_1(b - c)]^{-1}\{[k_1 N_0 - n_0(c + k_2 + K)] \times (b + k_2 + K) \cdot e^{bt} + [(b + k_2 + K)n_0 - k_1 N_0] \times (c + k_2 + K) \cdot e^{ct}\}, \qquad \textbf{3}$$

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where $b, c = \left(-a \pm \sqrt{a^2 - 4k_1K}\right)/2$, $a = k_1 + k_2 + K$, n_0 and N_0 are initial values at time zero, when the two are in equilibrium $N_0k_1 = n_0k_2$. For the working equation, we combine Equations 2 and 3, substituting n_0 and N_0 in terms of N_{total_0}.

$$N_{\text{total}} = N_{\text{total}_0}[(k_1 + k_2)(b - c)]^{-1}\left[-(a + b)(c + K)\cdot e^{bt} + (a + c)(b + K)\cdot e^{ct}\right] \quad \mathbf{4}$$

MATERIALS AND METHODS

Preparation of 601.2 mutants

A HaeIII recognition sequence was generated at a chosen locus by each of the eight mutations on pGem-3Z carrying the 601.2 nucleosomal DNA sequence.

The original 601.2 sequence is as follows:

5′-GGACCCTATACGCGGCCGCCCTGCAGAAGCTTGGTCCCGGGGCCGCTCA

ATTGGTCGTAGCAAGCTCTGGATCCGCTTGATCGAACGTACGCGCTGTCCC

CCGCGTTTTAACCGCCAAGGGGATTACTCCCTAGTCTCCAGGCACGTGTC

AGATATATACATCCTGTGCATGTA-3′

New HaeIII sites were then introduced individually by PCR mutagenesis as highlighted below on the sequence, and designated dyad to dyad-7 where the number indicates the number of helical turns from the dyad. In each case, the remainder of the sequence was unchanged except for the pre-existing HaeIII site. The positions of introduced sites correspond to averaged positions of maximum cleavage by hydroxyl radical of 601.2 DNA reassembled into nucleosomes.

dyad-7 dyad-6 dyad-5

5′-GGACCCTATACGCGGCCGCC**GGCC**AGAAGC**GGCC**TCCCGG**GGCC**GCTC

dyad-4 dyad-3 dyad-2 dyad-1 dyad

AA**GGCC**TCGTAG**GGCC**CTCTGG**GGCC**GCTTGA**GGCC**ACGTACG**GGCC**GTC

CCCCGCGTTTTAACCGCCAAGGGGATTACTCCCTAGTCTCCAGGCACGTGT

CAGATATATACATCCTGTGCATGTA-3′

Nucleosome reconstitution and restriction digests

DNA was ^{32}P-labeled at one end by means of PCR. Eight species were mixed in equal amounts prior to reconstitution of nucleosome core particle by means of gradual salt dialysis using purified chicken erythrocyte histone octamers (5). Typically, this resulted in a 120 µl aliquot containing ~50 nM nucleosome in 10 mM Tris–HCl, pH 7.4.

The enzyme reaction was carried out at 37°C by adding 1.2 µl of enzyme (at suitable concentrations) to 9.6 µl of reconstitute in a total volume of 12 µl containing 50 mM NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$, 20 µg/ml BSA and 5% glycerol. The concentration of glycerol was constant at all enzyme concentrations. An aliquot of 1 µl of sample was removed at defined time intervals, quenched with 9 µl of gel-loading buffer containing 50 mM EDTA, 50% formamide, 7 M urea and 0.05% xylene cyanol. Reaction products were resolved on 8% denaturing acrylamide gels, and quantified by using a PhosphorImager.

For reactions including HMG-D/Z and histone H5, 1 µl of protein was mixed with 8.6 µl of reconstitute to final concentrations specified in the legend to Figure 4 and 1.2 µl of 10× enzyme reaction buffer for a 15 min incubation period at 37°C before the addition of enzyme as described above.

Data analysis

Rate constants were obtained by the method of global curve fit (to Equation 4) combining results of different enzyme concentrations. The pair of constants, k_1, k_2 and K (scaled to enzyme level), were set as global parameters respectively for the same sites and enzyme concentrations, or otherwise as local parameters. Initial fits were set up in MicroSoft Excel Solver (7) and refined in the GraphPad Software Prism 4 where the best fit errors were calculated. The global method rules out the possibility of false solutions using one enzyme concentration alone, since reciprocal relations among rate constants can differ despite defining the same curve.

For simplicity in experiments with a fixed enzyme concentration studying the effects of HMG-D/Z and histone H5 on nucleosome binding, a single exponential decay was used for curve fitting instead of Equation 4. The apparent rate constants K_{ap} were normalized to the values of control experiments using nucleosomes alone. Standard deviations were calculated from repeated experiments.

Experimental considerations

The enzymatic reactions are set up so that $[n] \ll K_{\text{m}}$, as this simplifies the derivation in that $K = V_{\text{max}}/K_{\text{m}}$. It is most likely $k_2 \gg k_1$, maintaining a low level of effective substrate. Situations where $[n]$ is not strictly $\ll K_{\text{m}}$ reduce the accuracy of K alone, as now $K = V_{\text{max}}/(K_{\text{m}} + [n])$, but affect little the values of k_1 and k_2. Given these considerations, the substrate concentrations used in this study satisfy the kinetic requirements.

The biphasic characteristic of a degradation curve ought to be negligible due to minute amounts of n_0 ($k_2 \gg k_1$), but may be more apparent as a result of free DNA released from histone octamer upon dilution (8,9). This causes k_2 to be underestimated in curve fitting. For this reason, dilutions of reconstitutes in the final reaction mixture were minimized.

RESULTS

The implication of Scheme b is that proportionality can be seen between overall cleavage rate and low level of enzyme, having a low K counterbalancing a large k_2. This linear relationship breaks down at high enzyme concentrations, once the extremity of reaction rate (limited by k_1) is reached. Therefore for inner sites likely to have slower equilibrium (small k_2), maximum cleavage rate is reached with low enzyme concentration and remains constant beyond further increase in enzyme concentrations. In view of this, an appropriate range of enzyme concentrations consistent with both scenarios ensures accurate estimates of k_1 and k_2 by curve fitting.

A typical experiment is shown in Figure 1. Bands on a denaturing gel (Figure 1a) were quantified and presented in Figure 1b as decline of substrates in time. Unlike other sites, complete cleavage appeared instantaneously at dyad-7. In a different experiment of higher enzyme concentration, shown in Figure 1c, there was an apparent increase in the extent of cleavage at dyad-6, in comparison to sites nearer the dyad whose cleavage rates remained largely unchanged suggesting low accessibility at these sites.

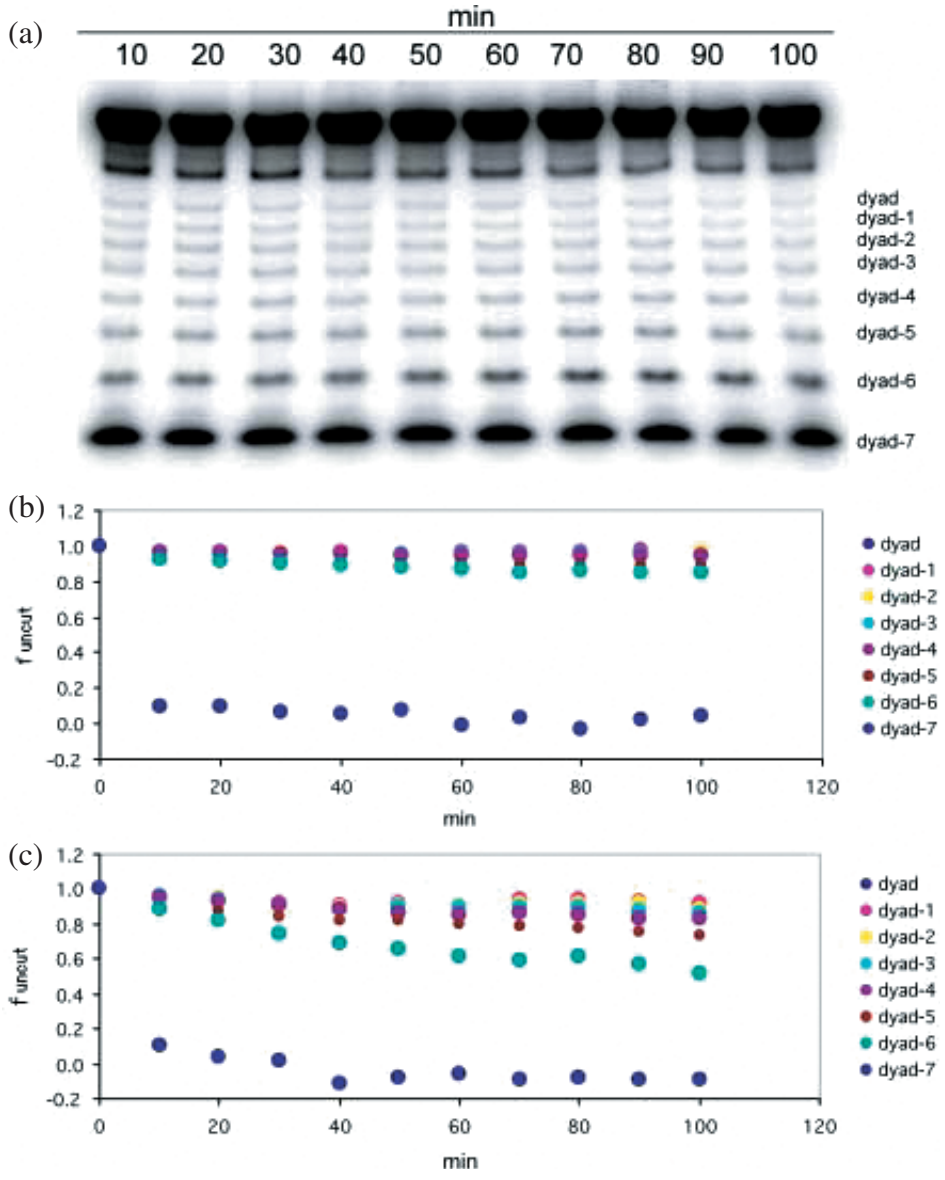

Figure 1. (**a**) A digestion time course of cleavage using 500 U/ml HaeIII. (**b**) Product bands from experiment shown in (a) were quantified and expressed as uncut fractions. (**c**) Parallel experiment with a 10-fold higher enzyme concentration.

Representative sets of theoretical curves were drawn in Figure 2, each fitted to data points from a single experiment using a fixed enzyme concentration. Pairs of k_1 and k_2 thus obtained for all sites were plotted in Figure 3. As discussed, sites at dyad-7 and dyad-6 were more accessible, having high k_1 and k_2 values.

Different digestion patterns were observed when each of HMG-D, HMG-Z or linker histones was bound before enzyme addition. Histone H1 protected against cleavage at the majority of sites most obviously at dyad-6, while in contrast HMG-D and to a lesser extent HMG-Z increased the extent of cleavage at dyad-5, dyad-6 and dyad-7. Figure 4 compares the normalized values of K_{ap} in the presence of the proteins with values in control experiment using nucleosomes alone. These observations confirm a previous report (5) that the two proteins tested have opposing effects on the accessibility of DNA exiting and entering the 601.2 nucleosome but differ from this report in the response close to the dyad. The increased accessibility induced by HMG-D at the dyad using the original Widom and Anderson assay (6) is also observed with other HMGB proteins (A. Ragab, unpublished data). We surmise that the different results with the two assays are intrinsic and may be related to the particular properties of the relevant restriction enzymes, e.g. differences in k_3 or k_p.

DISCUSSION

Validity of the assay

The aim of developing this assay is to simplify the original accessibility assay developed by Widom and co-workers

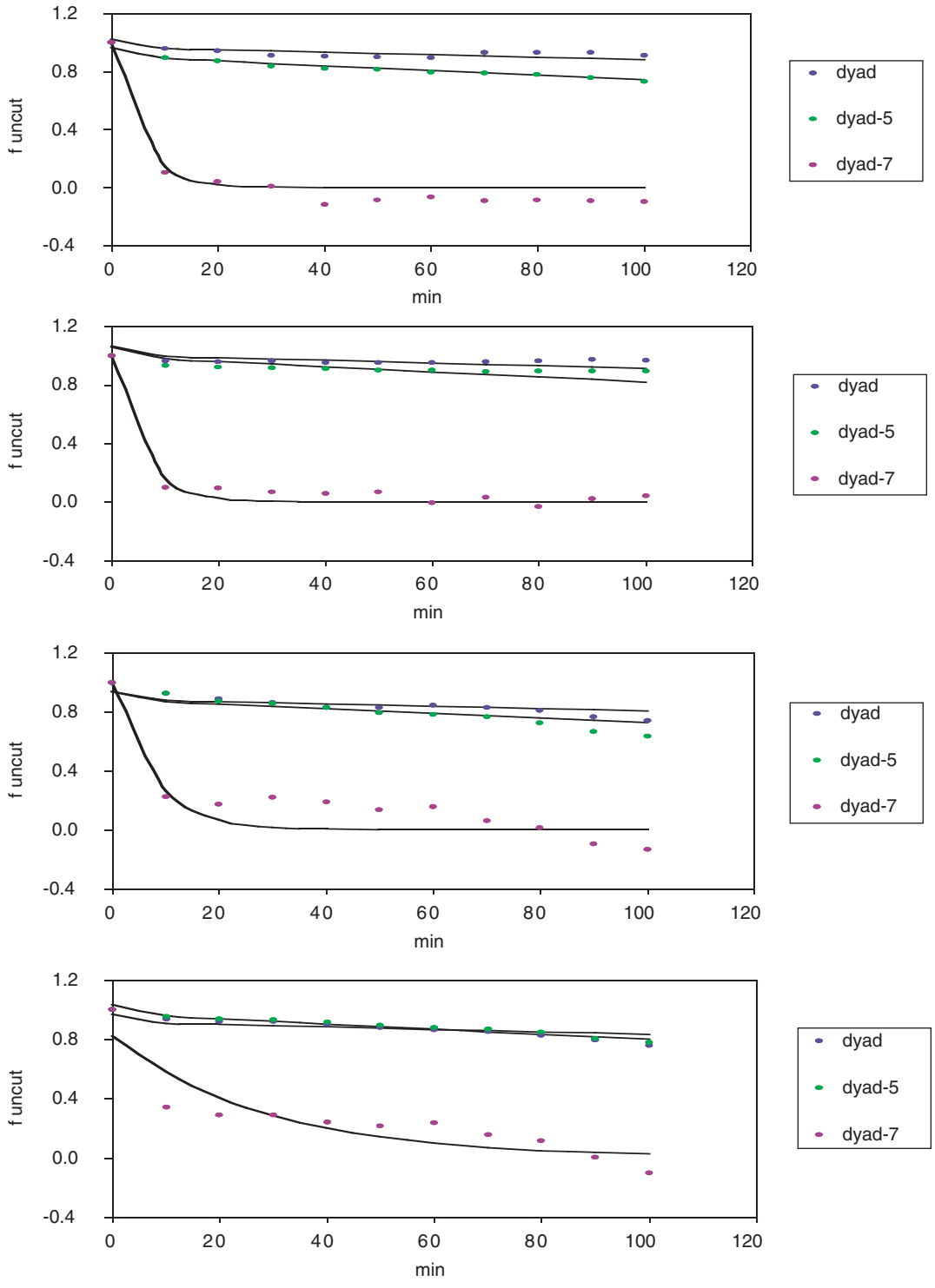

Figure 2. Parallel experiments (conditions identical to Figure 1) were conducted using four enzyme concentrations (from top to bottom: 5000 U/ml, 500 U/ml, 50 U/ml and 5 U/ml). For clarity, experimental data points as well as best fit curves are shown for three sites.

(1–4,6) and in particular to reduce possible influences of different rotational positioning of restriction sites and of different assay conditions and enzymatic mechanisms. The variability in assay conditions used for the cleavages of sites on a single reconstituted nucleosome is relatively small and was thought unlikely to greatly influence the interpretation of the results. However, the other variables are less easy to quantify.

By introducing the same restriction site at different positions, we have created eight different DNA sequences in place of the single one used by Widom and co-workers. The sequences are designed so that the HaeIII site always occupies

a rotational position with an outward-facing minor groove. The sequence of the HaeIII site, GGCC, is such that this position would be highly preferred (10). We therefore thought that it is extremely unlikely that any of the altered sequences would adopt a different rotational position. Indeed, the ExoIII digestion patterns of the original sequence and of the mixture of mutated sequences are essentially identical (data not shown). In some cases, for example to those at the dyad, the sequence changes are highly conservative. Another possibility is that the new sequences might differ significantly in affinity for the octamer from the original sequence. Again, we believe this to be unlikely because the new sequences retain identified important positioning signals at ±1.5 (11,12). Again, since the major histone–DNA contacts occur where the minor groove points in towards the histone octamer, the changes we have made, where the minor groove points out, should only influence affinity by altering the mechanical properties of the sequence. Most importantly, the results we have obtained are internally consistent, suggesting that there are no major effects of altered positioning or affinity.

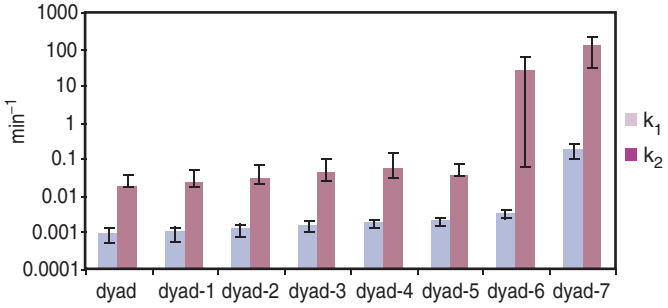

Figure 3. k_1 and k_2 obtained from experiments described in Figure 2 were plotted in pairs for all sites.

Local fluctuations of histone–DNA contacts

The data show that there is a sharp diminution in the rate of cleavage between sites −7 and −5, and therefore there is relatively little change in the overall rate between sites −5 and the dyad itself. This result is slightly different from that reported by

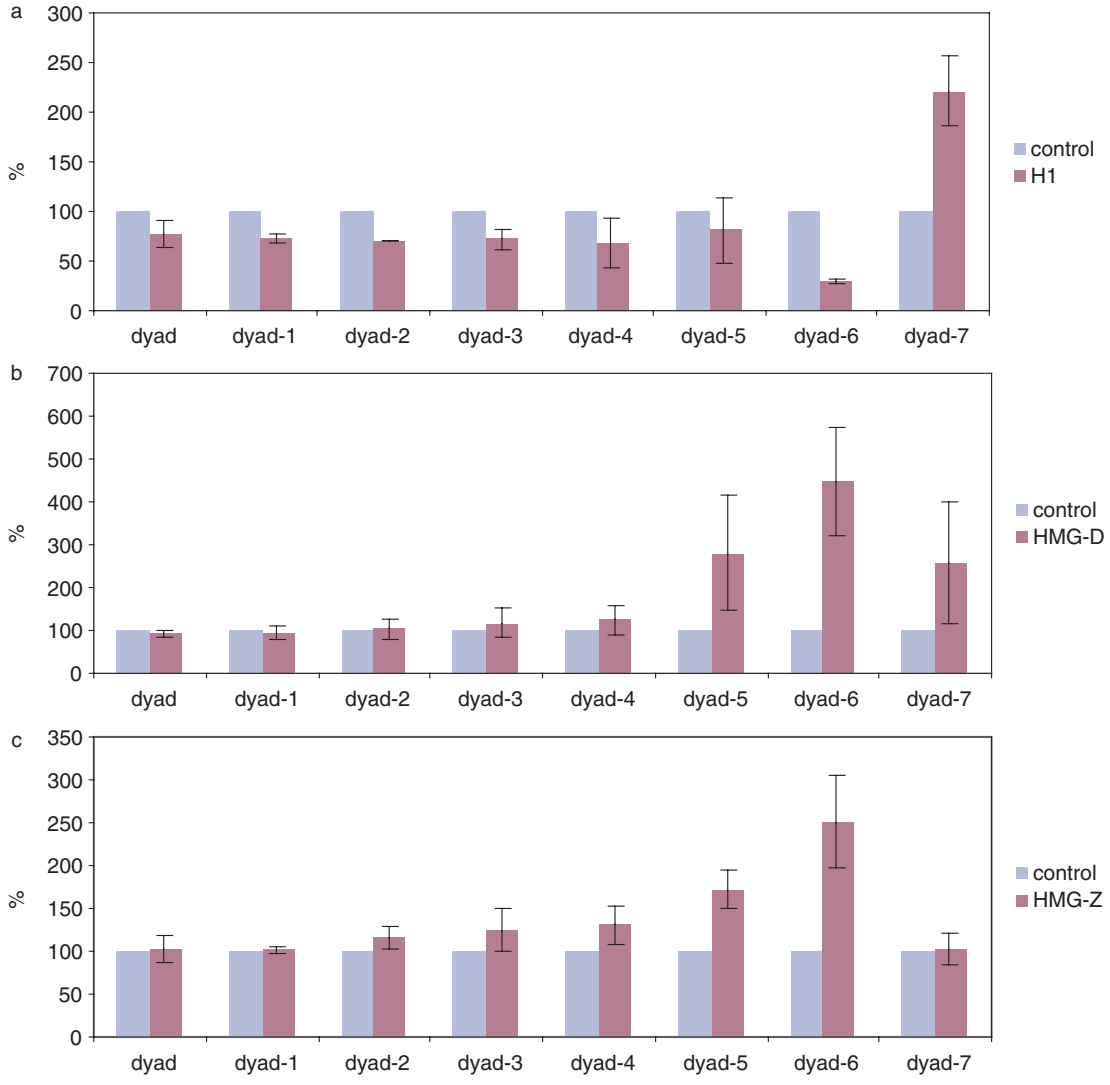

Figure 4. Effects of linker histone H1 (**a**), HMG-D (**b**) and HMG-Z (**c**) compared in terms of normalized apparent rate constants. The final concentrations for H1, HMG-D and HMG-Z in the reaction mixtures were 19 nM, 2.1 μM and 2.3 μM, respectively.

Anderson and Widom (4) who showed that between sites −5 and the minimum observed rate at site −1 there was a more substantial decrease in the cleavage rate. The initial sharp decline in the rate between sites −7 and −5 is wholly consistent with a transient unwrapping but it is more difficult to explain the plateau between −5 and the true dyad on this basis. One possibility is that in this region, which is dominated by interactions between the H3/H4 tetramer and DNA, local breaking and remaking of the histone–DNA contacts may be an additional factor determining accessibility. Such a possibility would be consistent with the binding of the transcription factor NF-κB to central positions in an apparently intact nucleosome (13) and could involve ligand-dependent changes in the binding of the basic histone tails, as invoked for the increased accessibility induced by HMG-D (5).

By simple simulation, a wide range of K_{eq} values suggests that the cleavage at internal sites is either difficult to detect or unmeasurably fast at either of the two moderate distant sites under identical conditions. Previous workers had not observed and therefore not discussed such phenomenon. In contrast, K_{eq}s (k_1/k_2) calculated from the present study show very similar values, consistent with a fluctuation event.

Altered accessibilities of nucleosomal DNA by linker histone and HMG-D/Z

Linker histones are believed to bind asymmetrically to nucleosome core particle making DNA contacts at dyad and entry/exit region (14–18). HMG-D and HMG-Z are two abundant proteins in embryonic *Drosophila*, and the former has been shown to compete with histone H1 in chromatin assembly (19,20). How these proteins could impose steric hindrance on DNA and/or (de)stabilize DNA-octamer contacts is not fully established. Decrease in accessibility in the presence of linker histone can be interpreted as lengthened DNA residence on octamer, for a high k_2 relative to k_1, or vice versa in the presence of HMG-D/Z. Observations in this study suggested that the DNA accessibility of neighbouring sites within the same nucleosome affected by these chromosomal proteins has immediate implications for a non-invasive mechanism in gene transcription.

Protein concentration influences nucleosome dynamics

A higher k_1/k_2 ratio is a general feature of experiments employing low substrate concentration (data not shown). Despite suspected errors caused by dissociation in diluted nucleosome samples, it is possible that this result reflects a real variation. Protein concentration will affect nucleosome dynamics, such that in a more diluted environment DNA could be on average more loosely wrapped. These observations may provide an explanation for the small value for k_2 of $\sim$2.6 s^{-1} for all sites in comparison to a predicted value of $\geqslant 10^5$ s^{-1} (1,2). Previous studies used high nucleosome concentrations (at least 100 nM total concentration) including reconstitutes with carrier DNA, which may result in a large k_2 maintaining the availability of nucleosomal DNA.

Comparison with the original method

The original method described by Widom *et al*. (1–3,6) approximated a single exponential decay for describing the nucleosome degradation, since the observed biphasicity was negligible. Here, we summarize this approach. By simple derivations two equations were given below alongside schemes of the nucleosome and free DNA digest.

$$N \underset{k_2}{\overset{k_1}{\leftrightarrow}} n \overset{K}{\leftrightarrow} P \quad N = N_0 \cdot e^{-\frac{2k_1K}{k_1+k_2+K+\sqrt{(k_1-k_2-K)^2+4k_1k_2}}\cdot t} \qquad \mathbf{5}$$

$$\mathrm{DNA} \overset{K'}{\leftrightarrow} P \quad D = D_0 \cdot e^{-K'\cdot t} \qquad \mathbf{6}$$

Accordingly, the observed rate constant for nucleosome degradation

$$K_{\mathrm{nucleosome}}^{\mathrm{observed}} = \frac{2k_1K}{k_1 + k_2 + K + \sqrt{(k_1 - k_2 - K)^2 + 4k_1k_2}},$$

which reduces to $K_{\mathrm{nucleosome}}^{\mathrm{observed}} = k_1K/K_2$ on two assumptions $k_2 \gg k_1$ and $k_2 \gg K$. In the case of free DNA $K_{\mathrm{dna}}^{\mathrm{observed}} = K'$. Division of the two yields

$$\frac{K_{\mathrm{nucleosome}}^{\mathrm{observed}}}{K_{\mathrm{dna}}^{\mathrm{observed}}} = \frac{k_1K}{k_2K'} = \frac{k_1}{k_2} = K_{\mathrm{eq}}, \text{ on a third assumption } K = K'.$$

Calculation of K_{eq} in this manner absolutely requires assumptions for each of the sites and enzymes to be validated, and consequently may be more prone to error.

CONCLUSION

We have designed a simple straightforward procedure to monitor variations in the accessibility of DNA bound to the histone octamer. The assay is sensitive to changes induced by the binding of the linker histone and of HMG-D/Z. The reactions occur under relatively mild catalytic conditions. This together with the ease of associated data handling permits the technique to be readily adaptable to other applications.

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