Relative Affinities of DNA Sequences for the Histone Octamer Depend Strongly upon Both the Temperature and Octamer Concentration

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ABSTRACT: Using a novel competition assay to determine the relative strength of different histone octamer-binding sites, we have compared three natural and two synthetic sites. We show that the relative affinities of these sites for the histone octamer depend upon both the temperature and octamer concentration. In particular, under certain conditions, a natural octamer-binding site from a yeast promoter outcompetes a synthetic sequence of comparable affinity to the strongest previously described positioning sequence. Under other conditions, this synthetic sequence is the preferred octamer ligand. We infer that sequence selection by the histone octamer depends strongly upon both the sequence-dependent anisotropy of DNA bending and on DNA deformability and that these parameters may contribute differently to nucleosome formation. These findings indicate that previous studies designed to identify strong octamer-binding sites may fail to select some natural strong binding sites.

The positioning of nucleosomes in promoter regions *in vivo* has often been proposed as an essential component of transcriptional regulation (*1*−*5*), yet the properties of many of these binding sites have not been systematically investigated. In contrast, the binding characteristics of both designed and selected deoxyribonucleic acid (DNA)[1] sequences with a high affinity for the histone octamer have been extensively described (*6*−*8*). However, at least one such high-affinity sequence (*6*) does not position efficiently *in vivo* (*9*, *10*). Comparing particular natural octamer-binding sites with selected and designed sites, we conclude that octamer binding to different DNA sequences depends upon both overall deformability and the anisotropy of DNA bending and suggest that different binding characteristics may be optimized for different biological functions.

The stability of the nucleosome core particle can be defined by the change in free energy upon DNA binding, $\Delta G = -RT \ln K_{eq}$. Various values of K_{eq} have been reported for bulk chicken erythrocyte chromatin as well as for defined DNA sequences (*11*−*14*). For relative comparisons, Shrader and Crothers (*6*) developed an approximate method of obtaining $\Delta\Delta G$, where knowledge of the absolute value of K_{eq} is unnecessary. This assay is based on each DNA competing with a common competitor over a limiting pool of octamer in parallel reconstitution experiments. The approximation is that $\Delta G = RT \ln K_{eq} - RT \ln K'_{eq}$, where equilibrium constants refer to $K_{eq} = [\text{nuc}]/[\text{DNA}]$ instead of by definition $K_{eq} = [\text{nuc}]/[\text{DNA}][\text{oct}]$. This treatment eliminates the term $RT \ln[\text{oct}]'/[\text{oct}]$ from the true $\Delta\Delta G$ value. This approximation is valid if free octamer concentrations are similar, for example, when the affinities are close or where the effective concentration of competitor DNA is very much greater than that of the tracer. In the latter situation, the tracer DNAs make little contribution because octamers are complexed mostly with the competitor, but detection of tracer DNA incorporated into the nucleosome is difficult.

Therefore, while the method of Shrader and Crothers (*6*) permits the assignment of a relative order of affinities, it is less informative in quantitative terms. To obtain more precise measurements of $\Delta\Delta G$, we have fluorescently labeled different DNA species so that their affinities for the histone octamer can be compared directly in one environment where all of the components equilibrate in the same pool of histone octamer. In this situation, the concentration of the octamer is eliminated during derivation, yielding $x = ye^{\Delta\Delta G_{xy}/RT}$, where x and y are the ratios of bound/free DNA for two different DNA sequences in the reaction mixture.

EXPERIMENTAL PROCEDURES

DNA Preparation. DNA stocks of ∼0.05−0.1 μg/μL were generated by means of PCR using fluorescent (fluorescein, Cy5, dye 782) labeled oligonucleotide primers. The nature of fluorescent labeling permits a simultaneous multisequence comparison. We have successfully assayed three DNA species, and addition of a ^{32}P-labeled DNA as a fourth species would also be straightforward.

Sequences used in this study were 601.2 (*7*), yeast CHA1 and PHO8 inducible promoters, *Lytechinus variegatus* 5S RNA gene (*1*), and a designed fragment (syn) containing a central 71-bp region retaining important positioning determinants but slightly modified from the palindromic consensus sequence described by Thåström et al. (*8*). The sequences used are listed below with phased TA or TG/CA steps in bold.

601.2, 5′-CTGCAGAAGCTTGGTCCCGGGGCCGCTCA-ATTGGTCGTAGCAAGCTCTGGATCCGCTTGATCGA-ACGTACGCGCTGTCCCCCGCGTTTTAACCGCCA-AGGGGATTACTCCCTAGTCTCCAGGCACGTGTCAGA-

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[1] Abbreviations: DNA, deoxyribonucleic acid.

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TATATACATCCTG-3′;
syn, 5′-GCTCTAGAACTAGTGGATCCCCCGGGC**TGCA**G-GAATTTAGCAAGCTCTAGGTGCGCTTAAAGTGTTCTAGA-GGCCCTT**TACA**GCCGTTTAAGCACTACTAGACGTTGTT-GAGCTTATCGATACCGTCGACCTCGAGGGGGGGCC-CGG-3′;
5S, 5′-CAACGAATAACTTCCAGGGATT**TATA**AGCCG-ATGACGT**CATA**ACATCCCTGACCCTTT**TA**AATAGCT**TA**-ACTTTCATCAAGCAAGAGCC**TA**CGACCATACCATGCTGA-A**TATA**CCGGTTCTCGTCCGATCACCGAAGTCAAGCAG-**CATA**-3′;
CHA1, 5′-GTGCCAAAGCTTTATCAAATTGTTTGCGAT-GAGATAAGATAAAAGGGACAA**TATG**AGGAGGAA-**C**ACAGG**TATATA**AATATCGCCAAATAAAAGGAAAA-TGTT**TATACA**GTTTTCTCTTTTTAGGTGCTGGATAG-ACAAGAGACAGGAAAATTAACCAGCGAGATGTCGATAGTC-**TACA**ATAAAACACC-3′;
PHO8, 5′-CCGTAATGCTGG**TATGTA**AATGTT**TATGT**
AGCCACTTGCTGGCAAAA**TGTG**GAGCACGAAAA**T**
ATATAAACGTTTAGA**TACA**CTTCTTTCTT**TATATA**-CTAAAATCCCTTTACTTTTCCTCTACGCGCGAAGC-TTATCGCTATCGTCAATGCTGCTATT**CATG**-TCGATGCTGCTCTTCCGTTAGACGCG-3′.

Nucleosome Reconstitution and Determination of $\Delta\Delta G$. A total of 0.4−3 μL of histone octamer stock solution (0.1 μg/μL) was added to 10 μL of DNA stock (two sequences) in a final volume of 30 μL in 20 mM Tris-HCl at pH 7.4 and 0.75 M NaCl. The amounts of DNA relative to one another affect only detectability. The mixture was incubated overnight at various temperatures as specified in captions of the figures, followed by dialysis for 1 h at 4 °C into low-salt buffer before quantification on a native acrylamide gel, run at 4 °C. A plot of x versus y was obtained by titrating DNA with octamer, and thereby, $\Delta\Delta G$ was determined using the function $x = y\Delta e^{\Delta\Delta G_{xy}/RT}$. Histone octamers were prepared from chicken erythrocyte nuclei by the method of Huynh et al. (*15*).

RESULTS

For this study, we chose three positioning sequences, the *L. variegatus* 5S sequence, 601.2, and syn sequences and compared these with two sequences from the yeast promoters for the PHO8 and CHA1 genes. These sequences correspond to positioned nucleosomes *in vivo* (*16*, *17*). The PHO8 and CHA1 octamer-binding sites contain the TATA box and unlike most previously described strong octamer-binding sites have a relatively low stacking energy (Table 1). The syn sequence has a comparable affinity to the highest previously reported affinity of any DNA sequence for the histone octamer (*8*).

The procedure of nucleosome reconstitution in this study is based on capturing the rapid histone−DNA equilibrium at moderate salt concentrations by a one-step dialysis to low salt at 4 °C. This approach proved to be comparable to the conventional gradual dialysis method. The level of reconstitution varies slightly with different initial salt concentrations. Given the complexity of the variation in histone−DNA interactions in relation to the ionic environment, we have not investigated this aspect further. Consequently, all reconstitutions were standardized by allowing the DNA and octamer to equilibrate overnight at 0.75 M NaCl.

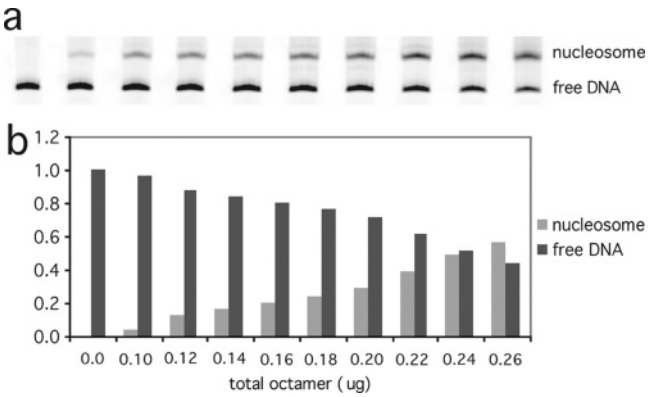

FIGURE 1: Nucleosome reconstitution of 601.2 DNA was performed at 4 °C as described in the Experimental Procedures using 0.5 μg of DNA. Reconstitute (18 μL) was mixed with 2 μL of 50% sucrose solution prior to analysis on a native acrylamide gel (a). Quantification is shown in b, plotting the relative amount of nucleosome and free DNA (normalized to the value of 1) in response to the amounts of added octamer.

To validate the assay, we titrated the DNA with increasing amounts of histone octamer. The quantification of nucleosomal and free DNAs for one such experiment (with a single sequence) is shown in Figure 1. For determination of $\Delta\Delta G$, the mixture of two sequences was titrated with an increasing octamer concentration, and at each octamer concentration, the nucleosomal/free DNA ratios of both sequences were obtained (quantified the same as in Figure 1) and fitted to the function $x = ye^{\Delta\Delta G_{xy}/RT}$ (see the Experimental Procedures). In the control experiment, two equivalent but distinguishable populations of 601.2 DNA were tested. Each population was labeled with a different fluorescent dye. We obtained, as expected, a $\Delta\Delta G$ value of 0 (Figure 2a). The same outcome was also obtained from a different control experiment, where reconstituted 601.2 nucleosomes were re-equilibrated in high salt with further addition of 601.2 DNA (labeled with a different fluorescent dye) (see the Supporting Information). The mixture was incubated overnight and dialyzed to low salt as described in the Experimental Procedures. We also compared the 5S and PHO8 sequences at 4 °C and again obtained a linear plot giving a $\Delta\Delta G$ value of 0.69 ± 0.09 kcal mol^{-1} (Figure 2b). In this experiment, the 5S sequence outcompetes the PHO8 sequence.

In contrast, we have also observed nonlinear relationships between x and y with an increasing octamer concentration. For example, for CHA1 versus syn at 45 °C (Figure 3), a curve was obtained indicating a progression of stronger CHA1 affinities relative to syn at increasing octamer concentrations. The plot therefore does not represent a single unique $\Delta\Delta G$ value but instead a varying series of $\Delta\Delta G$ values dependent upon the octamer concentration. Notably, at the highest octamer concentration, CHA1 outcompetes the syn sequence. A similar nonlinearity was observed for 601.2 versus 5S, CHA1 versus 601.2, and CHA1 versus 5S, as summarized in Figure 4.

Parallel experiments to those shown in Figure 4 revealed that lowering the temperature (4 °C for 601.2 versus 5S, 37 °C for CHA1 versus 601.2, and 26 °C for CHA1 versus syn) favors 601.2 and syn and discriminates against CHA1 and the 5S sequence. At 4 °C, CHA1 was outcompeted by 601.2 to a similar extent at all octamer concentrations tested,

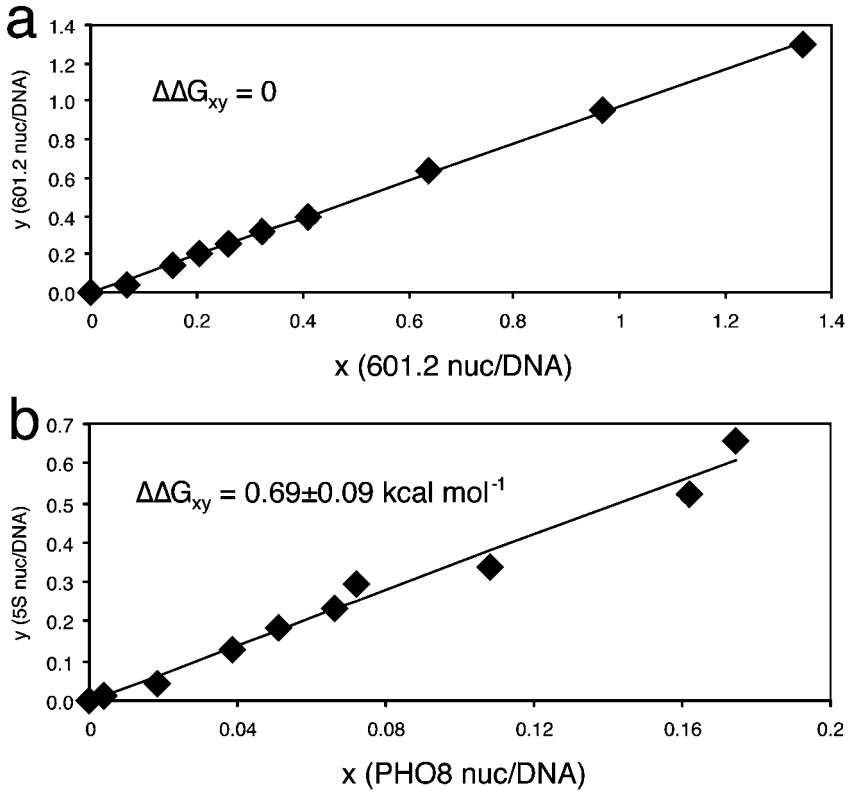

FIGURE 2: Comparison (b) of 5S and PHO8 DNA made at 4 °C. At each octamer concentration, the nucleosomal/free DNA ratio of one sequence was plotted against the other. $\Delta\Delta G$ values were obtained fitting the straight line $x = y\Delta e^{\Delta\Delta\Delta G_{xy}/RT}$, where $\Delta\Delta G_{xy}$ represents ΔG_{PHO8} to ΔG_{5S}. A control experiment (a) was carried out in which 601.2 DNA was compared to itself.

whereas at 37 °C, CHA1 outcompeted 601.2 at the highest octamer concentrations (Figure 5).

DISCUSSION

We have developed a method to compare directly the relative affinities of DNA sequences for the histone octamer and used this method to show that the outcome of a competition between different DNA sequences for the histone octamer depends very strongly upon the conditions of the assay.

Stein (*11*) derived an elegant method of acquiring K_{eq}, and following his work, an excellent refinement was made by Ausio et al. (*12*). K_{eq} reported by these authors reflects an average value of the bulk chromatin of chicken erythrocyte. K_{eq} values of defined DNA sequences have also been reported in more recent studies (*13*, *14*), in which the assumption was made that nucleosome assembly is a hyberbolic (or sigmoidal) function of the free octamer concentration. This approach is less preferable because accurate quantification of free octamer depends upon calculating a small difference between two large quantities. Further, the problem of aggregation caused by excess octamers limits exploration of the complete hyperbolic curve important for an analysis of this type.

One drawback of the reported method by Shrader and Crothers (*6*) is that changing experimental conditions (e.g., tracer DNA/competitor ratio or the competitor itself) alters the value of $\Delta\Delta G$ between two sequences. All K_{eq} values are temperature- and salt-dependent and therefore so are the resultant $\Delta\Delta G$ values. This study suggests that the K_{eq} of octamer−DNA equilibrium also depends upon the concentration of histones. These points are consistent with the variation in $\Delta\Delta G$ values observed in this work and possibly explain the variability of $\Delta\Delta G$ values reported in the literature. In the studies of Thåström et al. (*8*, *18*), on one occasion, $\Delta\Delta G$ values of sequences were obtained by means of dialysis (4 °C) to make a comparison with the stepwise dilution (at room temperature) originally employed by Shrader and Crothers (6). A ∼50% reduction was seen in all but one (15 of 16 sequences) measurement using the latter technique. Similar trends were noticeable in another instance when the competitor DNA was replaced. Furthermore, these studies have standard deviations averaging ±20% (dialysis) and ±87% (stepwise dilution).

The nonlinearity of the nucleosome-bound/free DNA ratios of the competing pair indicates that by interacting with the histones one sequence becomes a better octamer ligand relative to the other in response to high protein concentrations. This may explain the sigmoidal characteristics in the plots of the core particle versus the free octamer concentration in the studies of Thåström et al. (*14*), who defined such curves using the Hill equation. We infer that some sequences, depending upon physicochemical properties, have variable equilibrium constants for the histone octamer and therefore a pure hyperbola (Michaelis−Menten type) or that of the Hill equation, which may not be entirely appropriate at all times for the description of nucleosome assembly because the underlying mechanisms (both equations contain a single equilibrium constant, which in the latter case reflects the affinity of initial substrate binding to multiple sites) assumed for deriving these equations no longer apply.

Nucleosome assembly is influenced both by the local conformational space available to individual base steps, i.e., the flexibility/deformability, and by the configurational space

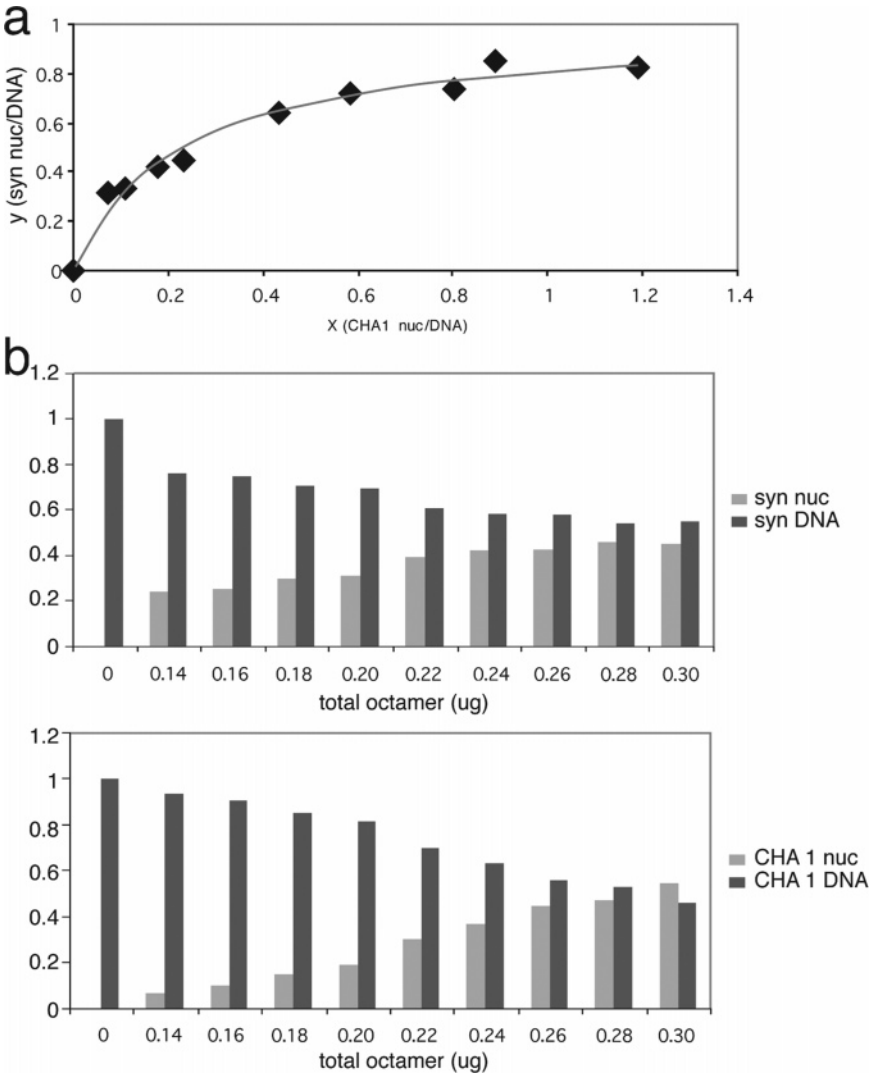

FIGURE 3: Analysis of CHA1 versus syn (a) identical to that of Figure 2 except for a different incubation temperature of 45 °C. (b) Titration profiles.

Table 1: Stacking Energies of Nucleosome-Binding Sites

	ΔG/*base step* (kcal/mol)
CHA1	−1.09
PHO8	−1.09
L. variegatus 5S	−1.14
syn	−1.17
601.2	−1.26
Saccharomyces cerevisiae telomere 3	−1.26
range for yeast	−1.03 to −1.28

[a] Values were calculated using a running 70-base step window. The figures for *L. variegatus* 5S, 601.2, and syn are the average for the central 70-base steps, while those for CHA1 and PHO8, which do not have a defined dyad, represent the average for the whole sequence. Note that the more negative the value the greater the stacking energy.

available to the bound sequence as a whole. The former facilitates the DNA distortion necessary to accommodate the overall curvature of the DNA wrapped on the surface of the histone octamer, while the latter specifies the ensemble of trajectories available to the DNA chain. In the context of competition between different DNA sequences, the sequence that most readily adopts the trajectory of the bound DNA will be favored on initial binding but the subsequent deformation will be favored by local deformability. The considerations imply that sequences of high anisotropic bendability will preferentially bind to the histone octamer at low octamer concentrations and at low temperature, where the restriction on configurational space is greatest. In contrast, higher temperatures promote the existence of high-energy species deviating from the preferred configurations with the consequence that the ranges of configurational space available to nominally isotropically or anisotropically bendable sequences become more equivalent. Any differences in the entropic penalty on binding are thus lower at higher temperatures so that the enthalpic contribution from deformability becomes more significant.

A simple measure of the overall deformability of a DNA sequence is the summed stacking energy. To calculate this parameter, we have used the values reported by Protozanova et al. (*19*). These values, unlike those of other compilations in the literature, correctly correlate with the experimentally determined persistence lengths of three strong octamer-binding sequences, CA, TATA, and NoSeq (AAT, unpublished observations). Of the sequences that we have used, CHA1 and PHO8 have the lowest overall stacking energy, while 601.2 has the highest (Table 1). The 601.2, *L. variegatus* 5S, and the syn sequences are characterized by phased TA (or CA/TG) steps interspersed with G/C-rich sequences of higher stacking energy. The positioning se-

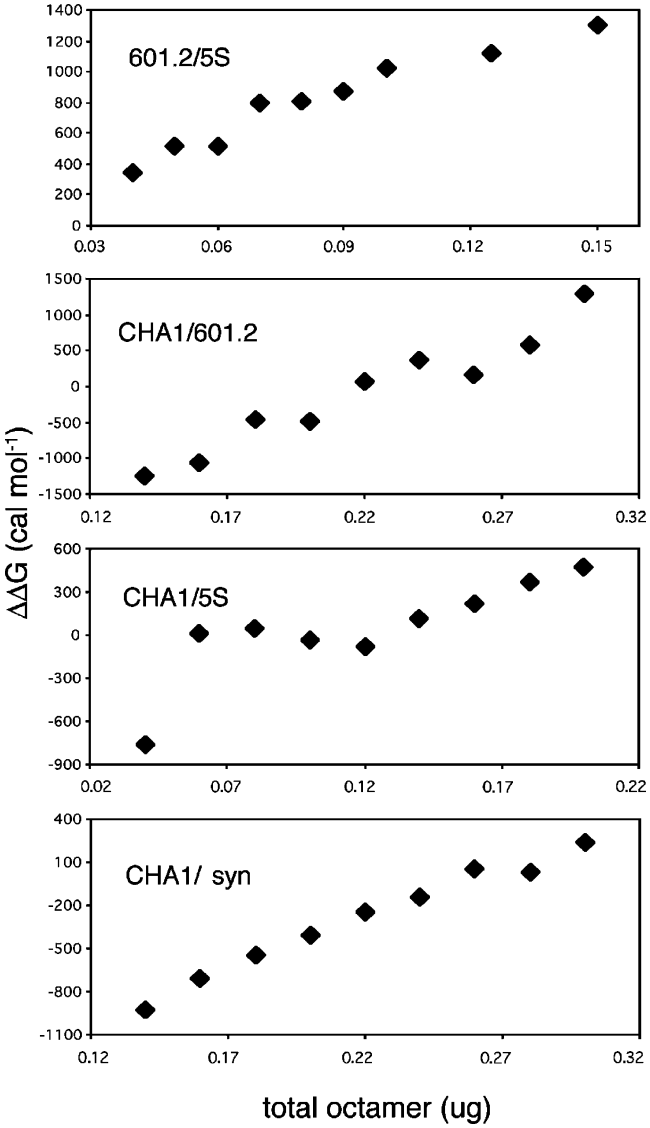

FIGURE 4: Series of $\Delta\Delta G$ values calculated in the experiment described in Figure 3, using, at each octamer concentration, the corresponding nucleosomal/free DNA ratios of the two sequences. Comparisons of other sequences [601.2 versus 5S (37 °C), CHA1 versus 601.2 (37 °C), and CHA1 versus 5S (37 °C)] were also carried out. Results were plotted against amounts of added octamer. $\Delta\Delta G$ values represent the difference B − A for the sequences A/B; for example, the values in Figure 3 were calculated as $\Delta\Delta G_{xy}$. A positive value shows that A outcompetes B, while a negative value shows that B outcompetes A.

quence designed by Shrader and Crothers (*6*) has similar properties with a stacking energy/base step of −1.20 kcal/mol. In all of these sequences, anisotropic bendability combines with high deformability, where the minor groove points toward the histone octamer to yield a sequence of high affinity and positioning power. In contrast, not only are the TA steps in the CHA1 and PHO8 sequences more random, but they are also more clustered, thus increasing the possibility of local bubble lesions, which in turn would promote hyperflexibility (*20*, *21*). We infer that, at high temperatures and high octamer concentrations, the greater deformability of the CHA1 sequence enables it to compete successfully with syn, a sequence which is anisotropically bendable but only locally deformable. The CHA1 sequence thus defines a different type of octamer-binding site. In contrast, the 601.2 sequence with a high average stacking

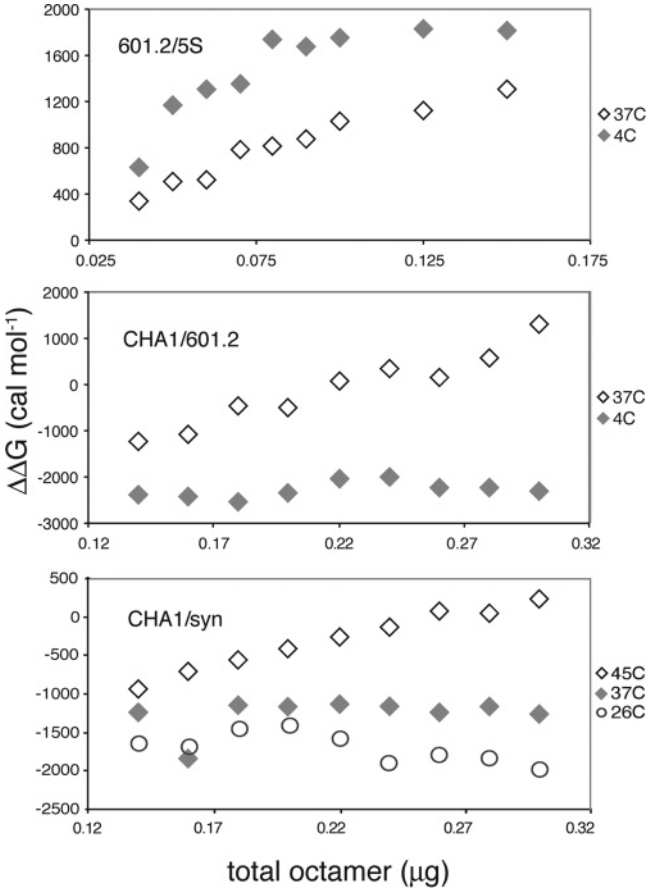

FIGURE 5: In the experiments of 601.2/5S, CHA1/601.2, and CHA1/syn described in Figure 4, three parallel experiments were carried out using lower incubation temperatures. The results are shown together with the Figure 4 data denoted by ◇.

energy is a stronger competitor at low temperatures (Figure 5).

RELATION TO PREVIOUS WORK

A number of different assays have previously been used to determine the affinity of a DNA sequence for the histone octamer. Typically, reconstitution reactions are performed under a standard set of conditions which, however, differ between studies. These variables include the temperature (for example, 4, ∼22, and 37 °C), the salt dilution protocol during dialysis, and the histone octamer concentration (see above). Consequently, the resultant $\Delta\Delta G$ values are not necessarily directly comparable. Similarly, selection for "high-affinity" octamer-binding sequences depends upon nucleosome formation under highly limiting octamer concentrations (approximately 10% of the DNA concentration) (*22*, *23*). Our results imply that these selection procedures would enrich for sequences with high anisotropy and phased deformability; i.e., they specify rotational positions but could select against more isotropically bendable but highly deformable sequences. Such an effect would be enhanced by selection at low temperatures. The selected sequences described by Satchwell et al. (*24*) are probably subject to similar constraints.

Of the high-affinity positioning sequences characterized *in vitro*, only those binding to the 5S rDNA of *Xenopus* and *Lytechinus* and to the MMTV promoter have been shown to position *in vivo*. Indeed, a designed anisotropically bendable sequence with a high affinity *in vitro* did not position

efficiently when tested *in vivo* (*9*, *10*). However, both the highly deformable CHA1 and PHO8 sequences define a region of preferential occupation by a histone octamer *in vivo*. We suggest that the relative importance of overall deformability and anistropic bendability for nucleosome positioning *in vivo* may depend upon the location of the nucleosome. Both *CHA1* and *PHO8* are inducible genes. On one hand, the high deformability of the octamer-binding sequences could enhance the stability of the nucleosomes once formed, and on the other hand, the adoption of a large ensemble of configurations in the free DNA could facilitate induction when the affinity of the octamer for DNA is reduced by modification of the histones.

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SUPPORTING INFORMATION AVAILABLE

Competition of 601.2 nucleosome with free 601.2 DNA. This material is available free of charge via the Internet at http://pubs.acs.org.

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