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Artificial nucleosome positioning sequences

(chromatin/histone–DNA binding/DNA bending)

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ABSTRACT **We have used the emerging rules for the sequence dependence of DNA bendability to design and test a series of DNA molecules that incorporate strongly into nucleosomes. Competitive reconstitution experiments showed the superiority in histone octamer binding of DNA molecules in which segments consisting exclusively of A and T or G and C, separated by 2 base pairs (bp), are repeated with a 10-bp period. These repeated $(A/T)_3NN(G/C)_3NN$ motifs are superior in nucleosome formation to natural positioning sequences and to other repeated motifs such as AANNNTTNNN and GGNNNCCNNN. Studies of different lengths of repetitive anisotropically flexible DNA showed that a segment of ≈40 bp embedded in a 160-bp fragment is sufficient to generate nucleosome binding equivalent to that of natural nucleosome positioning sequences from 5S RNA genes. Bending requirements along the surface of the nucleosome seem to be quite constant, with no large jumps in binding free energy attributable to protein-induced kinks. The most favorable sequences incorporate into nucleosomes more strongly by 100-fold than bulk nucleosomal DNA, but differential bending free energies are small when normalized to the number of bends: a free energy difference of only about 100 cal/mol per bend (1 cal = 4.184 J) distinguishes the best bending sequences and bulk DNA. We infer that the distortion energy of DNA bending in the nucleosome is only weakly dependent on DNA sequence.**

Since nucleosome formation can prevent the binding of regulatory proteins (1), the mechanism by which core histone binding sites are determined is of central importance to understanding gene regulation. Numerous authors have reported nucleosomes in regular arrays (phased nucleosomes) in the genome (2–5), and several contributing factors have emerged. One proposed cause is a boundary effect (6) in which a tightly bound nonhistone protein or histone–nonhistone protein complex (7) forms an immovable anchor around which nucleosomes must organize. A second proposed model suggests that higher-order folding of chromatin modulates the placement of nucleosomes (8). Finally, there may be mechanisms for ordering nucleosomes in arrays based on DNA sequence-dependent interactions within each nucleosome. These could take the form of low-level, periodic signals that facilitate DNA bending, and thus the curving of the nucleic acid around the histone octamer (9, 10), or more conventional recognition of a short DNA region through specific contacts to the individual histones. The above effects are not mutually exclusive, and regions of phased nucleosomes with both sequence-dependent binding and boundary effects have been reported (11, 12).

The sequence periodicities reported in the eukaryotic genome fall into two categories. In early work, Trifonov and Sussman (9) deduced periodicities in the eukaryotic genome that included repeats of the form AANNNTTNNN. The direction of bending of this motif has not been determined, but base-pair tilt (to yield bending toward the backbone rather than compression of a groove) has been proposed (9). This directionality results from symmetry considerations if AA and TT segments, separated by half a helical turn, are both to bend preferentially toward the bound core histones.

The second type of repeat to be reported, but one that has been characterized more thoroughly, consists of alternating A/T- and G/C-rich regions with a total period of 10 base pair (bp) (ref. 10; see also Fig. 1). These periodicities seem to exist over the entire ≈145 bp of the DNA protected in mononucleosomes, with some irregularities near the dyad (13). Correlation of minor-groove accessibility with sequence shows that these fragments preferentially orient in nucleosomes such that G/C regions are found where the major groove is compressed (faces in as the DNA axis curves around the protein); A/T sequences are favored at sites of minor-groove compression (10). These preferred orientations also reflect flexibility rules determined for DNA bending by the catabolite activator protein (CAP) of the *Escherichia coli lac* promoter (14), suggesting that they are not nucleosome-specific but result from sequence-dependent anisotropies in the bendability of DNA.

Along with these periodic sequence effects, there are several examples of specific nucleosome positioning sequences (15–17). The corresponding DNA fragments bind histones to form nucleosomes at defined positions *in vitro* and potentially could act as stable nucleosome boundaries *in vivo*. In at least one of these sequences the nucleosome occupies a position advantageous to the binding of a regulatory protein (17). Mutagenesis studies on some fragments of this type revealed an essential positioning region that is reasonably short (40–50 bp), but no specific signal has been identified (16, 18). Inspection of these molecules shows some alternating A/T- and G/C-rich regions at major- and minor-groove compression sites, but nonperiodic signals have also been proposed (19).

Natural nucleosomal DNA contains only modest sequence periodicity; our experiments probed the extent to which more powerful binding sequences can be designed. Additionally, we attempted to determine which of the experimentally determined sequence periodicities is most advantageous to histone binding and whether or not signals of this kind might be responsible for natural nucleosome positioning sequences. The strong nucelosome binding sequences we describe should be useful for further *in vitro* and *in vivo* investigation of the role of histone–DNA binding affinity in the packaging and function of genomic DNA.

EXPERIMENTAL PROCEDURES

Cloning Vectors. Two vectors, based on pGEM-2 (Promega), were used throughout this study. The majority of the fragments were made by cloning multimers of oligonucleotides into pGEM2-Ava. This vector was produced from pGEM-2 in two steps. First, the unique, symmetric *Ava* I site in the original vector was destroyed by cutting with *Ava* I, filling in the resulting overhangs with the Klenow fragment of

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DNA polymerase I, and reclosing. A new, asymmetric *Ava* I site (20) was then introduced into this intermediate plasmid by cloning the sequence GATCGCTCGGGTG into the *Bam*HI site. Due to the lack of restriction sites on the SP6 promoter side of the *Ava* I site of the pGEM2-Ava polylinker, a second vector was used for very short inserts. This chimeric vector was constructed by cloning the 1425-bp *Eco*RI–*Ava* I fragment of pBR322 into pGEM2-Ava. This new vector has a *Sty* I site ≈80 bp from the asymmetric *Ava* I site.

Oligonucleotide Inserts. Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer and purified by electrophoresis in 12–15% polyacrylamide gels containing 50% (wt/vol) urea in TBE (45 mM Tris/45 mM boric acid/1mM EDTA, pH 8.3). The purified single strands were phosphorylated with polynucleotide kinase and then annealed by slow cooling from 90°C to 4°C. Approximately 10 μg of this double-stranded oligonucleotide was polymerized for 15 min at room temperature, and the ligation mixture was fractionated in a 6% polyacrylamide gel run in TBE. The desired multimer band was visualized by staining with ethidium bromide, excised, and soaked overnight at 45–55°C. The samples were spun twice to remove gel slices and the DNA was precipitated with ethanol. Purified multimers were cloned into the *Ava* I site of the appropriate vector. Clones were screened by the alkaline lysis method and verified by dideoxy sequencing (technical manual, Promega).

Reconstitution, Binding, and Footprinting. The procedure used for reconstituting nucleosomes was similar to histone exchange methods used previously (16). The major differences were the addition of bulk competitor DNA and much lower levels of total protein. In brief, polynucleosomes stripped of histones H1 and H5 were produced by the standard methods (21) from chicken blood purchased from Pel-Freeze Biologicals. The material was checked for histone content and integrity, divided into aliquots, and frozen at −20°C for future use. In binding studies, ≈5 μg of this stripped chromatin was mixed with ≈20 μg of bulk DNA (isolated from chicken erythrocytes) and 0.1 μg of a labeled DNA fragment in 20 mM Tris/1 M NaCl containing 100 μg of albumin per ml and 0.1% Nonidet P-40 (Shell Chemicals, London). The mixture (70 μl) was incubated at room temperature for 30 min before the salt concentration was lowered to 0.1 M with three additions of 210 μl of 20 mM Tris buffer (20 min apart, room temperature).

The percentage of a DNA fragment that had been incorporated into nucleosomes was assayed by separating free DNA from complexed DNA in 5% polyacrylamide gels (75:1 acrylamide/*N*,*N*′-methylenebisacrylamide weight ratio) run at 20°C in TBE. The gels were dried and autoradiographed for 1–2 hr. The resulting films were used as templates to locate the radioactive bands, which were excised and quantitated in a Packard 1500 scintillation counter.

The free energy reported for a given sequence (Seq) was calculated from the equation $E(\text{Seq}) = RT\ln[\alpha(\text{TG})] - RT\ln[\alpha(\text{Seq})]$, where $\alpha(\text{TG})$ is the ratio of counts in the nucleosomal complex band for the pentamer of the TG reference oligonucleotide (see Fig. 1 and Table 1) to counts in the free TG pentamer band, and $\alpha(\text{Seq})$ is the analogous ratio for Seq reconstituted under identical conditions. Each fragment was reconstituted in two separate experiments and the results were averaged. The two numbers generally agreed to within ≈100 cal.

For hydroxyl-radical footprinting studies, the nucleosomes were produced by histone exchange without added competitor DNA. The salt was lowered from 1 M to ≈25 mM by slow dialysis. This resulted in >90% incorporation of the labeled fragments into nucleosomes. The hydroxyl-radical footprinting procedure was as described elsewhere (22).

RESULTS

Competitive Reconstitution Approximates an Equilibrium Distribution. Our experimental approach (Fig. 1) measures the ability of a labeled DNA fragment to compete with bulk DNA for a limited number of histone octamers during the nucleosome reconstitution procedure. The results are given as apparent free energy differences in the binding of a variety of sequences relative to binding of a standard reference sequence. It should be recognized that the partitioning of DNA molecules during binding in a salt gradient could, in principle, reflect either kinetic or equilibrium factors, or some combination of the two. Although most of the major points of this study depend on the ranking of sequences and would not change if the reconstitution efficiencies partly reflected a nonspecific, kinetic process, control experiments implied that the free energy differences closely approximate a true equilibrium and are probably relevant to nucleosome formation at physiological salt levels. Test reconstitutions reached the same final (labeled nucleosome/labeled free DNA) ratios regardless of whether the radioactive fragment was initially added as free DNA or in nucleosomal complexes. This convergence to common percentages of incorporation starting from either very high or very low fractions of labeled nucleosomes suggests a true equilibrium. Additionally, neither extending the incubation time in the high-salt buffer nor changing the rate at which low-salt buffer was added affected the fraction of nucleosomes produced, to

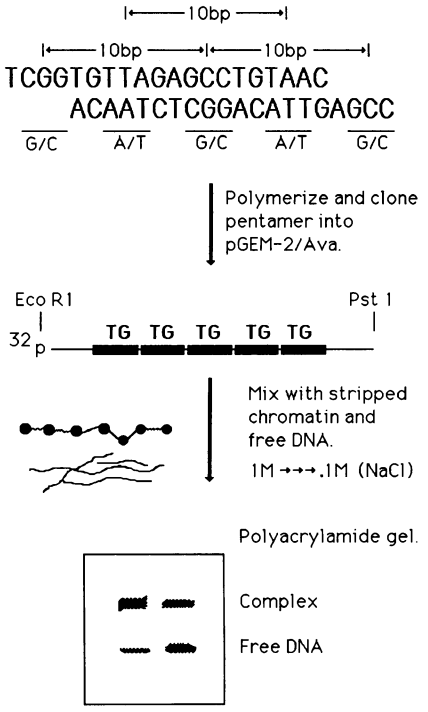

FIG. 1. Competitive reconstitution of the reference sequence. The reference used to define the zero point in binding free energy contains a direct repeat of five copies of the 20-bp "TG" oligonucleotide centered in a 163-bp DNA fragment. [Fragments containing 20-bp repeats bind more tightly than those containing 19- or 21-bp repeats (unpublished data).] This sequence contains alternating A/T and G/C regions with a total period of 10 bp. All other reconstitution efficiencies (binding free energies) are relative to this number. Positive free energies correspond to sequences that reconstitute less well than the reference sequence. The reconstitution procedure is similar to other histone exchange procedures except for the addition of bulk DNA to reduce the protein/DNA ratio. The (complex DNA/free DNA) ratio, and thus free energies of binding, is determined by quantitating the amount of radioactivity in the respective bands of a polyacrylamide gel.

within the reproducibility of these experiments (≈100 cal/mol). Finally, both the persistence length of DNA and the sedimentation coefficient of the nucleosome are nearly independent of salt concentration in the range 0.1–1 M NaCl (23, 24). Thus the DNA complexed with histones at high salt is tightly folded and subjected to similar bending stresses as DNA in a nucleosome at physiological salt concentration. This argues that the differential binding energies of two sequences ($\delta\Delta G$) at 1 M and 0.1 M salt would be very similar. However, the negligible histone exchange rates make the low-salt experiment impossible.

Some Synthetic Sequences Bind More Tightly Than the Natural Sequences. The results of competitive reconstitutions of several synthetic and naturally occurring nucleosome positioning sequences are shown in Table 1. The data suggest several main points. First, molecules based on alternating blocks of A/T- and G/C-rich regions bind histones quite well. The two most efficient nucleosome-forming sequences, code-named TG and GT, are examples. These fragments differ only by two nucleotides in the segment between A/T- and G/C-rich blocks where neither groove faces the protein and sequence changes are expected to have small effects on bending (13, 14). Chemical footprinting experiments confirmed this orientation (Fig. 2). Note that the relative binding free energies for the sequences TG and GT are more favorable than those found in the natural positioning sequences by about 1.4 kcal/mol; the natural sequences are, in turn, superior to bulk nucleosomal DNA by a similar margin.

Second, sequences that are repetitions of the AANNNTTNNN or GGNNNCCNNN motif (TR-5 and TRGC) are relatively poor binders among the periodic sequences. This result implies that the periodicity in which A/T and G/C blocks alternate is more favorable to nucleosome formation than is the AA. . .TT periodicity originally deduced (9). Along with the rough bending equivalence of AA and TT segments described earlier (13, 14), this supports models in which bends occur primarily by roll between adjacent base pairs at the loci where the grooves face the protein core.

Table 1. Comparative reconstitution free energies

Sequence	Energy, cal/mol
Designed	
TG (TCGGTGTTAGAGCCTGTAAC)$_5$	≡0
GT (TCGGGTTTAGACCTTGTAAC)$_5$	≈0
TG-T (TCGGTGTCAGAGCCTGTGAC)$_5$	1950
TR-5 (TCGGAAGACTTGTCAACTGT)$_5$	1900
TRGC (TCGGACTCCAGAGGTCACCA)$_5$	1300
Natural	
Lytechinus variegatus 5S RNA gene (SIM)	1250
Xenopus laevis somatic 5S RNA gene (XLS)	1350
Xenopus laevis trace oocyte 5S RNA gene (XLT)	1600
Mononucleosomal DNA (≈250 bp)	2850

Free energies of reconstitution were measured for five designed sequences, each containing a pentamer of an oligonucleotide, and three natural nucleosome positioning sequences. TG and GT are nearly equivalent except for the inversion of a dinucleotide in a nonbending region. TG-T is similar to TG but with interrupted A/T regions. TR-5 is a sequence based on out-of-phase repetitions of AA and TT. TRGC is analogous to TR-5 but based on GG and CC. All energies were calculated relative to the TG pentamer. Lower free energies correspond to a higher tendency to reconstitute. All designed sequences were excised from pGEM2-Ava as *Eco*RI–*Pst* I fragments. SIM is a 207-bp fragment isolated from an *Ava* I digest of p5S207-18 (25). XLS and XLT are, respectively, a 220-bp *Bam*HI–*Rsa* I fragment from pXP-1 and a 200-bp fragment *Rsa* I fragment from pXP-8 (≈pXlt400) (26). Mononucleosomal DNA was isolated from nuclei digested with micrococcal nuclease and was quite heterogeneous in size.

Finally, a sequence in which the A/T regions have been interrupted with a G or a C (TG-T) binds histones with intermediate strength. This result, along with the relatively weak binding of sequences TR-5 and TRGC in which only A/T or G/C blocks are provided, suggests that both the A/T and the G/C regions contribute to differential binding free energies.

A 40-bp Segment of Repetitive DNA Gives Binding Affinity Equivalent to the Natural Positioning Sequences. To determine how much repetitive DNA is required to provide the binding strength of the natural nucleosome positioning sequences, we constructed a series of fragments in which different numbers of the GT oligonucleotide were centered in fragments of overall constant length. This gave sequences with the same total amount of DNA but varying amounts of anisotropically flexible DNA. The results of reconstitution of these sequences is shown in Fig. 3. The two natural sequences that have been shown to position nucleosomes, SIM (15) and XLS (17), bind nucleosomes about as tightly as two copies of the GT oligonucleotide centered in a 170-bp fragment. The trace oocyte 5S RNA gene fragment, for which less is known about nucleosome placement, binds less well. Hence, a binding signal of the size of the natural nucleosome positioning sequences can easily be contained in about 40 bp of repetitive DNA.

Bending Contributions Are Reasonably Constant Over the Nucleosome. Fig. 3 summarizes the binding free energies as a function of the size of the insert of anisotropically flexible DNA: as its length increases, the energy of binding increases steadily. These data reveal no large jumps in the binding energy that might be ascribed to the tight kinks seen in the nucleosome crystal structure (27) and reflected in DNA cutting patterns (28). The largest energy gap found (660 cal/mol), between trimer and tetramer, results from the poor binding of the trimer fragment. Due to the location of restriction sites in the original vector, the oligonucleotide-based portion of the trimer sequence is near one end of the molecule. This does not allow a histone octamer centered on the flexible region to make all the possible contacts with the DNA and lowers the relative binding free energy for the fragment. The result is that the trimer reconstitution energy is anomalously close to that of the dimer (and thus far from that of the tetramer).

There is no evidence for a switch in the optimal phasing of flexible regions over the central DNA turns at the nucleosome dyad, as suggested to be the case from nucleosome sequencing experiments (13). Such a switch, reflecting a "key ring" structure, would be expected to lead to a marginally favorable or even unfavorable contribution to binding energy as the anisotropically flexible DNA segment length passes the critical size of the region of the protein that requires altered phasing (we presume that this would occur upon the addition of the second GT oligonucleotide). Instead, the increase in binding energy due to additional anisotropically flexible oligonucleotides is approximately constant. Independent evidence, based on molecules containing permuted sequences of different oligonucleotides, suggests a small increase in binding requirements very near the dyad (unpublished data).

DISCUSSION

Our results show that it is possible to mimic the binding energy of natural nucleosome positioning sequences by using short regions of repetitive DNA. Furthermore, longer stretches (120 bp) of these repetitive motifs yield DNA fragments that are stronger histone binders than the natural sequences (by a margin similar to the difference between the natural positioning sequences and bulk nucleosomal DNA). Our choice of specific A/T and G/C regions was essentially

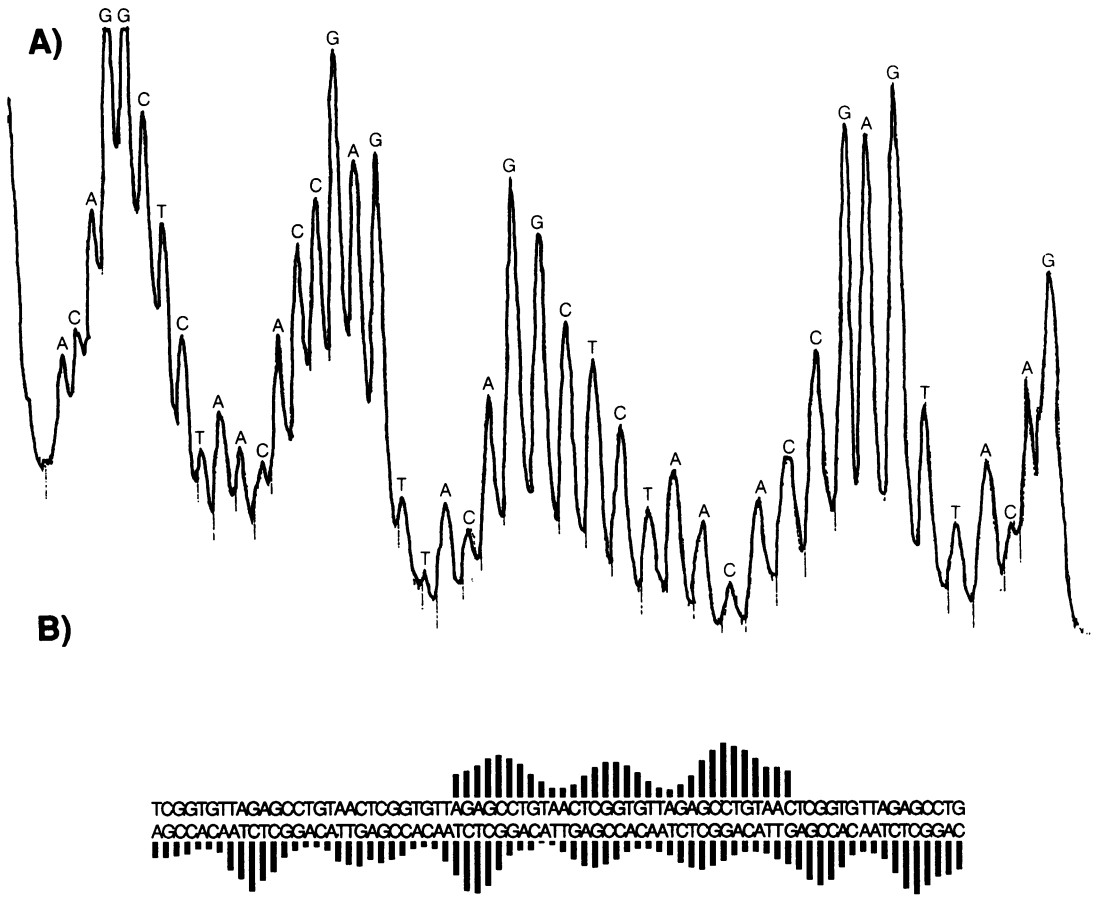

FIG. 2. Hydroxyl-radical cutting. (*A*) Densitometer scans of autoradiographs of the cutting pattern of 3′-labeled TG-5 fragment complexed with histones. These scans are highly structured with the G/C regions near the cutting maxima. The strikingly preferred rotational orientation suggests highly anisotropic bending properties for the DNA fragment. Band assignments were made by comparison to a lane containing the products of a cytosine-specific Maxam–Gilbert sequencing reaction. (*B*) Schematic of the cutting on opposite strands. The length of each bar is proportional to the cutting at that site. The stagger of the cutting maxima between strands in indicative of minor-groove cutting. As expected, the G/C regions are oriented with their minor groove facing away from the protein and are consequently cut most strongly. The integrated area of each band was normalized by dividing by the peak area, and thus the cutting intensity, for the same band in a free-DNA reaction. This reduced the effect of sequence-specific variations. The normalized values were averaged with their 5′ and 3′ neighbors before plotting (three-bond running average).

arbitrary, as was the DNA in the nonbending regions, making it improbable that we have arrived at the best possible histone-binding sequence. Nonetheless, these sequences are significantly tighter nucleosome binders than 5S RNA genes, suggesting that the natural sequences contain positioning signals that are small and whose binding strengths are far from the maximum levels that could be achieved.

The rotational orientation of TG-like sequences is quite specific, but the translational positioning is less exact. Exonuclease III studies of the nucleosome formed from a fragment with five copies of the TG oligonucleotide revealed at least two sets of pause sites (unpublished data). This is the expected finding due to the moderate energies of replacing vector DNA with one anisotropically flexible oligonucleotide segment (see Fig. 3). Relative energies of this size (≈450 cal) suggest that the occupancies of the optimal position and a position 10 bp (half a segment) away should vary by less than a factor of 2.

The intermediate binding strength observed for natural nucleosome positioning sequences is of potential significance for dynamic processes involving nucleosomes. Our results show that in comparison to molecules designed using simple periodic rules, natural nuclesome positioning sequences are good but not optimal histone binders. The modest binding strength of the natural sequences implies that an important part of positioning a nucleosome may be the ability to move or remove it.

Also noteworthy is the small total range of the binding free energy observed for the sequences studies. In contrast to current work on prokaryotic protein–DNA interactions (29–31), where the ability of a DNA sequence to form a distorted phosphate backbone conformation is thought to be a major determinant of its binding strength, widely divergent sequences seem to be incorporated into nucleosomes with only small differences in binding free energy. The difference between our best binding sequences and bulk nucleosomal DNA is roughly 3 kcal/mol; when this energy is averaged over 14 helical turns or 28 bend points, the differential effect is roughly 100 cal/mol per bend. Assuming that other binding energies are constant, we can ascribe the differential energy to DNA flexibility effects. It seems likely that nucleosomes are designed to be flexible enough to accommodate a variety of local distortion geometries, all of which produce nearly equivalent curvature of the bound DNA. The comparative weakness of the differential binding energies is probably too small to be the sole origin of ordered nucleosome regions *in vivo* and is consistent with current data suggesting an interplay of several factors in nucleosome phasing (8, 11, 32).

Finally, we have described the design of short, repetitive sequence motifs that may be used to position nucleosomes with variable affinities, reaching levels significantly greater

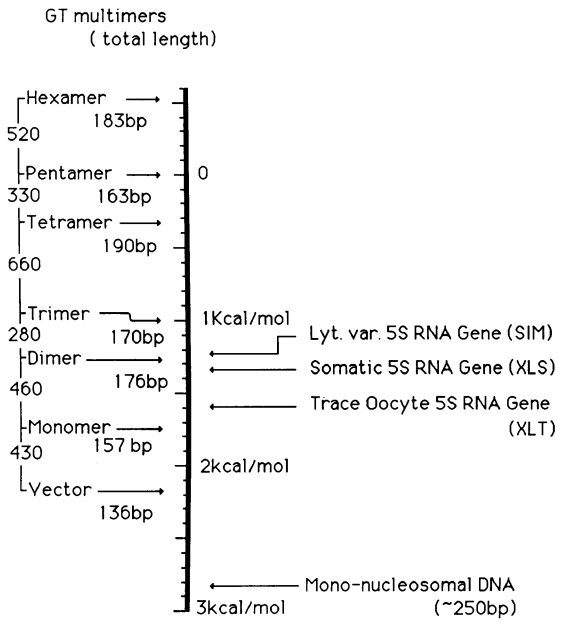

FIG. 3. Nucleosome binding as a function of the length of flexible DNA. The free energies of reconstitution of three natural nucleosome positioning sequences (see Table 1) are compared with those of designed sequences containing various lengths of flexible DNA. The binding of the best natural sequences can be mimicked with ≈40 bp of repetitive DNA. Additionally, the binding free energy increment for each additional flexible region is quite constant. The total length of each multimer (vector DNA plus the indicated number of 20-bp GT oligonucleotides) is given below the multimer name. The difference in binding free energy between fragments (cal/mol) is given at the extreme left. The monomer and dimer fragments were isolated from a *Sty* I–*Pvu* II digest of the chimeric vector (see *Experimental Procedures*), whereas the trimer through hexamer fragments were produced from pGEM-Ava. Due to a lack of appropriate restriction sites in the vector, the trimer oligonucleotide was somewhat asymmetrically arranged in the overall fragment so that it reconstituted anomalously poorly. The vector fragment contained the pGEM2-Ava polylinker and reconstituted significantly better than random DNA. These free energies are all relative to the GT pentamer, which binds histones with essentially the same affinity as the TG pentamer.

than those so far documented. In the future, constructs incorporating these sequences should prove powerful in further defining the importance of nucleosome binding in both gene regulation (3–5) and transcription (33–35).

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