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Differential Binding of c-Myc and Max to Nucleosomal DNA

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The ability of a transcription factor to function in vivo must be determined in part by its ability to bind to its recognition site in chromatin. We have used Max and derivatives of c-Myc to characterize the effect of changes of dimerization partner on binding to nucleosomal DNA templates. We find that homo- and heterodimeric complexes of these proteins bind to the CACGTG sequence in free DNA with similar affinities. Although Max homodimers bind to nucleosomes, truncated c-Myc homodimers do not. Surprisingly, modifying the c-Myc dimerization interface or changing its dimerization partner to Max enables nucleosomal DNA binding. Thus, changes in dimer structure or dimerization efficiency can have significant effects on nucleosome binding that are not predicted from their affinity for free DNA. We conclude that domains other than the basic region per se influence the ability of a transcription factor to bind to nucleosomal DNA and that changes of dimerization partner can directly affect the ability of a factor to occupy nucleosomal binding sites.

In eukaryotes, transcription occurs in an environment in which DNA is associated with histones and packaged into chromatin (36). The first level of packaging is the nucleosome core particle, which consists of a histone octamer (two H2A-H2B dimers and one H3-H4 tetramer) and 146 to 160 bp of DNA wrapped approximately two turns around the octamer (21, 37). In addition to assisting in the compaction of DNA into the chromosome, histones are believed to play a role in modulating genetic activity by inhibition of transcription (18, 33, 51).

Most transcriptional activators must bind to specific DNA sequences to function, and it is believed that activators frequently compete with histones for binding to DNA. The ability of a transcription factor to recognize a particular free DNA sequence in vitro does not necessarily mean that it will bind to this sequence when it is incorporated into nucleosomes (3, 26). Recently, investigators in several laboratories have examined the nucleosome-binding properties of several different transcription factors: whereas the glucocorticoid receptor (4, 39, 40), GAL4 (48, 56), and TFIIIA (25–27, 34) bind to nucleosomal DNA, heat shock factor (HSF) (48) and nuclear factor (NF1) (5, 41) are unable to bind to nucleosomes. Binding of certain transcription factors to nucleosomal DNA has been postulated to directly or indirectly alter nucleosome structure, enhancing the accessibility of promoter or enhancer sequences in chromatin (4, 5, 40, 41) or allowing subsequent entry of factors whose binding is normally blocked by nucleosomes. This alteration in local chromosome structure has been proposed to be important in the regulation of several eukaryotic genes.

An activator attempting to bind to nucleosomal DNA must contend with the relatively constrained structure of the DNA as it wraps around the nucleosome, and with contacts between the DNA and the histones that might compete for interactions required for binding by the activator. Nucleosomal binding can depend on a number of different factors: the rotational positioning of the binding site on the nucleosome (i.e., facing towards or away from the histones [4, 40, 41]); translational positioning on the nucleosome (i.e., position relative to the dyad axis and to linker DNA [25, 27]); acetylation status of the histones (10, 34); or an intrinsic ability of the particular activator to interact with nucleosomal DNA (48). These mechanisms are probably not mutually exclusive. In this paper, we begin to examine the role of DNA binding and dimerization domains in determining the ability of a transcription factor to bind to a nucleosome.

Transcription factors which belong to the basic-leucine zipper (b-ZIP) and basic-helix-loop-helix (b-HLH) families bind to DNA as homo- and heterodimers, with each monomer contributing a DNA binding domain and a dimerization interface. In b-ZIP proteins, changes of dimerization partner have been shown to affect the strength of binding to free DNA, DNA bending, and transcriptional activation (31). If individual DNA binding domains differ in their intrinsic ability to bind to nucleosomal DNA, then the combination of different monomers might specifically affect binding to nucleosomal DNA relative to free DNA. The hypothesis that we test here is that changes of dimerization partner will alter the abilities of b-HLH-ZIP proteins to bind to nucleosomal DNA. In order to investigate this issue, we have examined the abilities of c-Myc and Max dimers to bind to nucleosomes.

The c-*myc* oncogene is involved in cell proliferation and in inhibition of cellular differentiation (reviewed in references 8, 13, 17, 29, and 43). While it appears that interactions with Max (7, 9, 30, 42) are central to the mechanism of c-Myc function, the precise mechanism by which c-Myc regulates transcription remains unclear. c-Myc and Max offer an attractive system for characterizing the effects of changing dimerization partner on nucleosomal binding. Since Max homodimers and c-Myc/Max heterodimers bind preferentially to the same hexanucleotide DNA sequence, CACGTG (6, 22, 38), we can compare the abilities of different homo- and heterodimers to bind to a single site in a single position on a nucleosome. Any difference in the affinities of binding must result from structural differences between the protein dimers, not the position of the binding site. Finally, characterization of the abilities of c-Myc and Max to bind to nucleosomes is presumably relevant to the function of all b-HLH factors.

Using both electrophoretic mobility shift assays (EMSA)

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and footprint titration assays, we demonstrate in this study that changes in dimerization interface and dimerization partner significantly alter the affinity of binding to nucleosomal DNA but do not alter the affinity of binding to naked DNA. These data rigorously establish that changes in protein structure alter the ability to bind to nucleosomal DNA. They further imply that changes of dimerization partner can result in significant changes in the ability of heterodimeric regulatory proteins to bind to nucleosomal binding sites in vivo.

MATERIALS AND METHODS

Protein preparation. Polyhistidine-containing truncated c-Myc$^{342-439}$ and Max (54) and truncated c-Myc · GCN4 (14) were prepared as described previously and purified by affinity chromatography over a nickel chelate column (Qiagen) (1). Bacterially produced full-length c-Myc protein (53) and phosphorylated, baculovirus full-length c-Myc protein (38) were prepared as described previously. All proteins were stored in storage buffer (10 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol). Protein purity was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15% acrylamide) under reducing conditions. Estimated molecular weights of Max and truncated c-Myc monomers are 21,000 and 15,000, respectively. Activity of the preparations was determined as described below.

c-Myc/Max heterodimer purification. Heterodimers were purified by sequence-specific DNA affinity chromatography. CACGTG-containing oligonucleotides were concatamerized and conjugated to CNBr-activated Sepharose 4B (Pharmacia) (58). c-Myc/Max heterodimers were formed (15-min incubation at 43°C under conditions of c-Myc excess) and loaded on the affinity column. The column was washed with 3 to 4 volumes of column buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.9], 50 mM KCl, 0.075 mM EDTA, 1 mM dithiothreitol, 0.01% Nonidet P-40, 5% glycerol, 0.1 mg of insulin per ml). After rinsing with 4 volumes of 0.4 M KCl in column buffer, active heterodimers were eluted with 4 volumes of 1 M KCl in column buffer. The purity and relative activity of individual fractions were determined by EMSA (as described below) and standard SDS–15% PAGE under reducing conditions. Active fractions were pooled and dialyzed overnight (Spectrapor CE MWCO 2000) against storage buffer or column buffer.

DNA probes. A DNA probe containing a c-Myc/Max binding site separated by 23 nucleotides from an artificial nucleosome positioning sequence was created by annealing two 85-bp oligonucleotides with complementary 3′ 14-bp overlaps (85-E, 5′-GAATTCGTGATACGAGCCGGAAGCATAAAGTG AAAGCCTGGGGTGCCTAATGAGTGAGCTAACCA TGG**GACCACGTGCTC**CTGC-3′; 85-Xm, 5′-GGATCCCCC GGGGTTACAAGGTCTAAACCCGAGTTACAAGGTCT AAACCCGAATTACGCCAGATCTCCATGGCAGGAG CACGTGG-3′) and filling in with Klenow DNA polymerase to create a 156-bp double-stranded DNA molecule. The optimal c-Myc/Max binding site (38) is in boldface type, and the nucleosome positioning sequence, a tandem repeat of the 20-bp GT sequence known to induce rotational phasing (45), is underlined. The 156-bp fragment was amplified by PCR using the primers PCR-E (5′-ATATCGAATTCGTGATACGA-3′) and PCR-Xm (5′-ACTAGTGGATCCCCCGGGGT-3′), and the resulting 167-bp fragment was digested with *Eco*RI and *Xma*I and subcloned into a similarly digested pBluescript SK$^+$ vector (Stratagene) to create phBlue. The sequence of the insert was verified by DNA sequencing. To prepare nucleosomes lacking the CACGTG site (Nuc0), a similar fragment was isolated from phMLT, a plasmid identical to phBlue except that 22 bp containing the c-Myc/Max binding site was replaced by the TATA site from the adenovirus major late promoter (5′-GTTCCTGGGGCTATAAAAGGGG-3′). For EMSAs, the 154-bp *Eco*RI-*Bam*HI fragment of phBlue was gel purified, then double-end labelled by Klenow DNA polymerase fill-in with [α-^{32}P]dATP and [α-^{32}P]TTP, with the addition of unlabelled nucleotides for a subsequent 15-min chase. Labelled probes were separated from unincorporated nucleotides by using nick columns according to the manufacturer's (Pharmacia) instructions. For the single-end labelling required for DNase footprinting, the *Eco*RI-*Xma*I fragment was excised from phBlue, labelled by Klenow fill-in of the *Xma*I site with [α-^{32}P]dCTP and unlabelled dGTP, and gel purified. The large quantities of probe needed were obtained from 250-μl PCR reaction mixtures containing 650 ng of phBlue, 4 mM primers (PCR-E and PCR-Xm), 200 μM deoxynucleoside triphosphates, 2 mM $MgCl_2$, and 5 U of *Taq* polymerase in PCR buffer (Promega). PCR was performed for 30 cycles at 94°C (30 s), 55°C (30 s), and 72°C (20 s), with a final 10-min 72°C elongation step. DNA products were extracted with chloroform, phenol-chloroform–isoamyl alcohol, and chloroform-isoamyl alcohol and then ethanol precipitated and quantitated by electrophoresis and UV wavelength absorbance.

Nucleosome assembly. Gradient-purified nucleosomes were prepared by the dilution method of Perlmann and Wrange (40). Labelled probe (200 to 2,000 ng) was resuspended in 10 μl of 15 mM Tris (pH 7.5)–0.2 mM EDTA–2 M NaCl along with a 10-fold excess of nonspecific DNA (ϕX174 *Hae*III digest; Bethesda Research Laboratories) and 1.4 μg of HeLa histones (H2A, H2B, H3, and H4 prepared by the method of Stein and Mitchell [47]) per μg of total DNA. After a 20-min 37°C incubation, the reaction mixture was diluted at room temperature to 0.1 M NaCl in stepwise fashion over several hours by the addition of 10 μl of 15 mM Tris (pH 7.5)–0.2 mM EDTA (no NaCl) at 10-min intervals. The 200-μl sample was layered on top of a 5 to 30% glycerol gradient containing 50 mM Tris (pH 7.5)–1 mM EDTA and 0.1 mg of acetylated bovine serum albumin (Bethesda Research Laboratories) per ml and spun for 18 h at 32,000 rpm in a Beckman SW 55 rotor. Fractions (175 to 250 μl) were collected and analyzed by direct loading of 2.5- to 5-μl aliquots onto native 0.01% Nonidet P-40–5% polyacrylamide gels run in 0.5× TBE buffer (1× TBE is 90 mM Tris [pH 8.3]–64.6 mM boric acid–2.5 mM EDTA). The fractions containing the peak of nucleosomal DNA were pooled and applied to a second 5 to 30% gradient and resedimented under the conditions described above. Fractions containing the peak of unassembled DNA were pooled, extracted first with phenol-chloroform–isoamyl alcohol and then with chloroform, and ethanol precipitated for use as free DNA in footprint titrations. Double-purified peak nucleosomal fractions typically contained less than 5% unassembled DNA as judged by quantitation of EMSA gels on a series 400 Phosphorimager (Molecular Dynamics). Both unincorporated DNA and nucleosomal DNA fractions were stored at 4°C in gradient buffer.

EMSA. EMSA reactions were performed in a volume of 5 to 7.5 μl and included only proteins and labelled DNA (i.e., no carrier nonspecific DNA, such as dI:dC). After preincubation of proteins for 15 min at 43°C followed by a 5-min equilibration at room temperature, 2 pg of labelled DNA (either unincorporated or incorporated into nucleosomes) was added to the protein and incubated at room temperature for an additional 20 min. In nucleosome stability experiments, supercoiled pBluescriptIIKS$^-$ (Stratagene) was added in the indicated

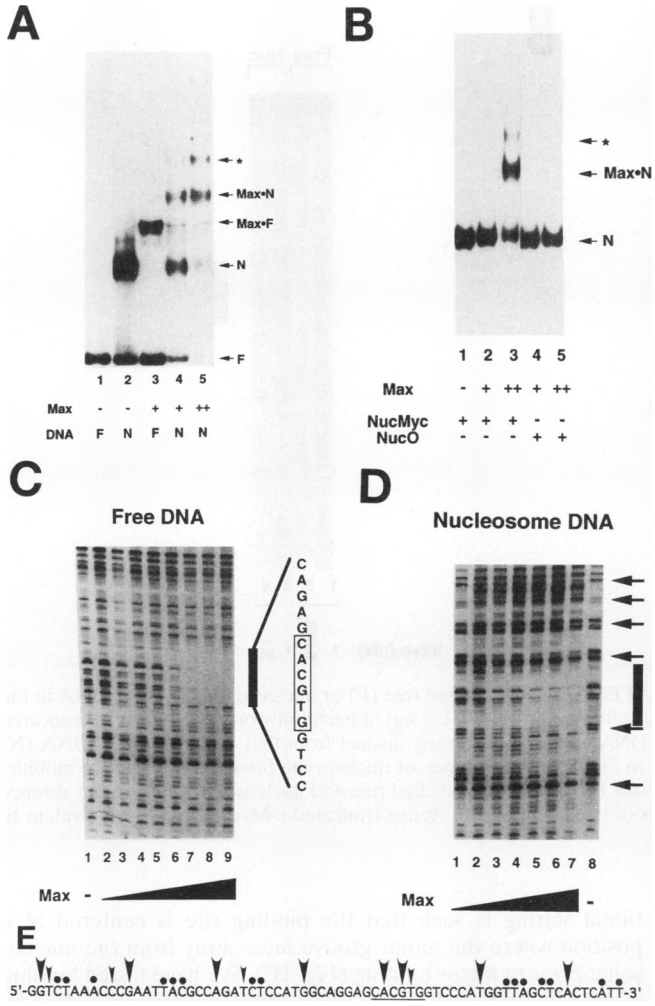

FIG. 1. Max binds both free DNA and nucleosomal DNA. (A) EMSA using labelled free (F) or nucleosomal (N) DNA in the absence (lanes 1 and 2) or presence (lanes 3 and 4, 3 ng; and lane 5, 4.5 ng) of bacterially produced purified Max protein. Complexes of Max with free DNA (Max · F) or with nucleosomal DNA (Max · N) are indicated. *, nucleosomes with more than one Max dimer bound. All samples were run on the same gel, but lane 1 was spliced adjacent to lane 2 for presentation. (B) EMSA using nucleosomes containing (NucMyc; lanes 1 to 3) or lacking (Nuc0; lanes 4 and 5) the CACGTG site, without (lane 1) or with (lanes 2 to 5) bacterially produced Max protein. The presence of a faint band in lane 5 at the Max · N position suggests that a small amount of nonspecific DNA binding by Max takes place, a finding consistent with footprinting at high protein concentrations (see panel D). All samples were run on the same gel, but lane 3 was spliced adjacent to lane 4 for presentation. (C) DNase I footprint with labelled free DNA and the following concentrations of Max (in nanomolar concentration of active dimer): lane 1, 0; lane 2, 0.038; lane 3, 0.078; lane 4, 0.15; lane 5, 0.31; lane 6, 0.70; lane 7, 1.4; lane 8, 2.8; and lane 9, 9.3. The position of the binding site and adjacent protected bases is indicated by the solid bar, with the core binding site boxed. (D) DNase I footprint with labelled nucleosomal DNA and the following concentrations of Max (in micromolar concentration): lane 1, 0.028; lane 2, 0.28; lane 3, 1.4; lane 4, 2.8; lane 5, 7.0; lane 6, 12.6; lane 7, 38; and lane 8, 0. Max at 1 μM is equivalent to 4.2 μg of protein added to the 100-μl reaction mixture. The position of the binding site and adjacent protected bases is indicated by the solid bar, and arrows indicate sites of enhanced cleavage in nucleosomal DNA separated by areas of increased protection. (E) Representation of the DNA sequence with bases whose cleavage is enhanced following assembly into a nucleosome with greatly enhanced (large arrowheads) or enhanced (small arrowheads) cleavage and bases with reduced cleavage by DNase (dots). The core CACGTG sequence is underlined. Note the 10-bp periodicity in the cleavage patterns.

amounts for an additional 5-min, room temperature incubation. Samples were loaded directly onto 0.01% Nonidet P-40–5% polyacrylamide gels which had been preelectrophoresed for a minimum of 30 min (15 to 20 *V*/cm) at 4°C in 0.5× TBE buffer containing 0.01% Nonidet P-40. Gels were run for 3 to 4 h, until a bromphenol blue marker (added to one or more lanes) reached the gel bottom. The presence of bromphenol blue did not affect complex mobility. Following electrophoresis, EMSA gels were dried and autoradiographed.

DNase I footprinting. Binding reactions were performed in volumes of 100 μl, containing 100 pg of specific probe (free or nucleosomal DNA) and the indicated amounts of protein, with the remainder of the volume being column buffer. Reaction mixtures were incubated for 20 min at room temperature before the addition of 5 μl of DNase mixture ($MgCl_2$ and $CaCl_2$, to achieve a final concentration of 3 mM each, and RQ1 DNase I [Promega] diluted to deliver the indicated number of units). DNase treatment for nucleosomes was 1 U for 2 min, and treatment for free DNA was 0.1 U for 1 min (100 pg of specific probe) or 3 min (1.1 μg of specific probe). Reactions were stopped, and the products were analyzed on 8% acrylamide–8 M urea sequencing gels. Gels were fixed in 5% acetic acid–5% methanol, dried, and exposed to XAR film or storage phosphor screens for quantitation on a series 400 Phosphorimager (Molecular Dynamics).

Determination of active protein. DNase reactions to determine active dimer concentrations were performed as described above but contained 1.08 μg of specific DNA (consisting of 1 to 1.5 ng of labelled 154-bp probe and 1.08 μg of cold 167-bp PCR product). The concentration of specific DNA in the reaction mixtures was therefore 1×10^{-7} M, approximately 100-fold greater than the dissociation constants of the proteins being assayed. Under conditions where the specific DNA is in such excess, all available dimers capable of binding will be bound. The moles of probe bound therefore equal the moles of active dimers. Individual proteins were titrated, and the percent footprint was calculated by standard methods (11). Points where less than 50% of the probe was bound were chosen to ensure conditions of DNA excess. It should be noted that the relationship between the concentration of active dimers and actual protein concentration is not the same for different proteins; this parameter is dependent on dimerization strength and stability, i.e., it reflects the likelihood of monomer association. Thus, 31 ng of truncated c-Myc will shift approximately the same amount of labelled probe as 3 ng of Max (compare Fig. 1A, lane 3, with Fig. 2A, lane 2). The total protein concentrations and percent active molecules for the preparations used to measure binding constants were as follows: p21 Max, 3 mg/ml, 100% active; truncated Myc-GCN4, 1.2 mg/ml, 9% active; and truncated Myc, 1.1 mg/ml, 4.5% active. All heterodimer preparations were fractionated by DNA affinity chromatography immediately prior to footprint titration assays and were 100% active.

Calculation of dissociation constants. For determination of dissociation constants, DNase reaction mixtures contained 100 pg of 154-bp probe in 100-μl volumes, equivalent to 1×10^{-11} M, approximately 100-fold less than the apparent K_ds determined. Under conditions where probe concentration is significantly lower than the apparent K_d, the percent occupancy of the binding site is a function of the apparent K_d. Each footprint titration was quantitated as described, and the concentration of

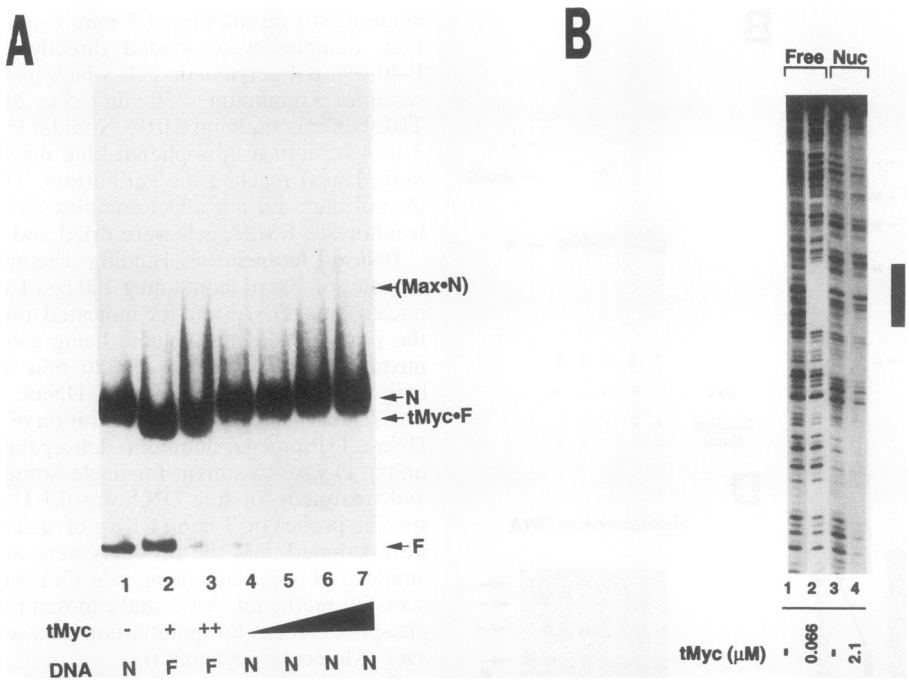

FIG. 2. Truncated c-Myc binds free DNA but not nucleosomal DNA. (A) EMSA using labelled free (F) or nucleosomal (N, Nuc) DNA in the absence (lane 1) or presence (lanes 2 and 4, 31 ng; lanes 3 and 5, 62 ng; lane 6, 94 ng; and lane 7, 125 ng) of bacterially produced purified truncated c-Myc protein. The mobility of the complex of truncated c-Myc with free DNA (tMyc · F) is clearly distinct from that of nucleosomal DNA (N) on a shorter exposure (data not shown); a longer exposure is shown here to illustrate the absence of nucleosome binding. The relative mobility of the Max-nucleosome (Max · N in parentheses) complex is shown. (B) DNase I footprint with labelled free and nucleosomal DNA in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of the indicated concentrations of truncated c-Myc. Active truncated c-Myc at 1 μM is equivalent to 49 μg of total protein in the 100-μl reaction.

active protein corresponding to the half-maximal binding was recorded as the apparent K_d determination for the individual experiment.

RESULTS

Nucleosome assembly conditions result in formation of mononucleosomes. The 154-bp DNA probe contained a single copy of the CACGTG binding site and two tandem copies of a 20-bp rotational phasing sequence (45, 46). The rotational phasing sequence is believed to induce a curve in the DNA; this favors a consistent orientation of the DNA in assembled nucleosomes. The length of the template allows formation of a single core nucleosome, with the CACGTG sequence located approximately at the dyad axis of symmetry. Nucleosomes were assembled by salt gradient dilution with purified core histones and were isolated by glycerol sedimentation gradients. Assembled nucleosomes migrate distinctly from free DNA in native polyacrylamide gels (Fig. 1A, lanes 1 and 2). Two independent experimental approaches were used to confirm that mononucleosomes were formed. First, digestion of the histone-DNA complex with micrococcal nuclease yielded a protected fragment of approximately 145 bp (data not shown), the expected length of DNA in a core nucleosome. This occurred at concentrations of micrococcal nuclease which completely digested mock-assembled probe. Second, when treated with DNase I, the assembled probe showed the 10-bp repeat pattern of protections and enhanced cleavages expected for rotationally phased DNA wrapped around core histones (arrows in Fig. 1D). This pattern reflects the alternating accessibility of the minor groove to DNase as it faces towards or away from the core histone surface. In the experiments presented, the rotational setting is such that the binding site is centered at a position where the minor groove faces away from the nucleosome prior to factor binding (Fig. 1E). We have tested binding of Max homodimers to two other rotational settings (offset by +4 and −5 bp) by DNase footprinting and see no significant effect of changing rotational setting on specific binding (data not shown).

Measurement of binding to nucleosomes. The DNase I footprint titration assay was used to measure the apparent dissociation constants (K_ds) of the various protein dimers for their binding sites in free DNA or in in vitro-assembled nucleosomes. In order to make a determination of the apparent K_d, it is critical to know the precise concentration of active (i.e., able-to-bind-DNA) dimer in each protein preparation. This parameter was determined by quantitating the relative amount of free DNA bound (percent footprint achieved) by increasing amounts of each of the various protein preparations in assays where the concentration of specific DNA was 2 orders of magnitude above the estimated apparent K_d (probe excess). Under these conditions, virtually all of the active dimers are expected to bind DNA. This method measures the equilibrium concentration of active dimers, and therefore should take into account the stability of dimerization, with the caveat that dimerization might be significantly affected by probe concentration. To determine the apparent K_d, the protein concentration was varied in assays where the concentration of DNA (free or nucleosomal) was at least an order of magnitude below the apparent K_d measured (limiting probe concentrations). The concentration of active dimers required to bind 50% of the DNA under these conditions provides an accurate measurement of the apparent K_d (2, 11, 28, 44). Note that in the

TABLE 1. Dissociation constants for dimers binding to the CACGTG site in naked and nucleosomal DNA

Protein	K_d for DNA (mean ± SEM)		Footprint	EMSA shift
	Free[a]	Nucleosome[a]		
Max	$(5.3 \pm 0.9) \times 10^{-10}$	$(2.5 \pm 1.6) \times 10^{-6}$	+	+
Truncated c-Myc	$(1.4 \pm 0.6) \times 10^{-9}$	ND[b]	−	−
Heterodimer	$(1.5 \pm 0.4) \times 10^{-9}$	ND	+[c]	+
Truncated c-Myc · GCN4	$(1.0 \pm 0.4) \times 10^{-9}$	$(1.1 \pm 0.7) \times 10^{-7}$	+	+

[a] Apparent dissociation constants are expressed as molarity. Experiments with Max and heterodimers were performed three and four times, respectively; experiments with truncated c-Myc and c-Myc · GCN4 were performed three and two times with free and nucleosomal DNAs, respectively. Only those titrations achieving saturation are included.

[b] ND, not determined because of a limitation in protein (see Results).

[c] Partial footprint.

footprint titration data presented below, amounts of added protein are presented as micromolar concentrations of active dimer (not total protein added) to facilitate comparison of the data and the measured K_d values. The relationship of this value to the total protein added varies as the activity of the specific preparation varies, as described in the figure legends.

We used EMSA in a qualitative fashion to assess whether there were differences in the abilities of homo- and heterodimers of c-Myc and Max to bind DNA. The DNase I footprinting protocol was used to confirm the specificity of binding and to quantitate differences in binding to nucleosomes. Both assays gave concordant results concerning relative differences in the abilities of the homo- and heterodimers to bind to nucleosomal DNA, demonstrating that these differences are reproducible in two very different assay protocols. Not surprisingly, the quantitative behaviors of c-Myc and Max homo- and heterodimers differ in these two assays. Whereas concentrations of homo- and heterodimers required to bind to a nucleosomal template are approximately 10^{-8} M in the EMSA, the DNase I footprinting protocol requires concentrations in the 10^{-6} to 10^{-7} M range (see the data below). Possible reasons for these quantitative differences include the following: (i) different solution conditions are required for each protocol (DNase footprinting requires conditions that allow DNase function); (ii) DNase footprinting measures binding in solution at room temperature, while EMSA measures complexes that are stable after electrophoresis for 2 h at 4°C; and (iii) the time-dependent nature of DNase I cleavage means that footprinting may be affected by rapid exchange rates of binding and may not reflect the instantaneous concentrations of protein-DNA complexes, resulting in higher apparent K_ds. Furthermore, the quantitative behavior of these proteins in the EMSA appears to be quite sensitive to assay conditions; depending on the amount of nonspecific DNA present in the reaction mixture, the amount of protein required to observe a mobility shift band ranges from 1 to 5,000 ng (data not shown). Since the EMSA was not intended to be used in a quantitative fashion, the footprinting-derived apparent K_d values should be used for comparative purposes. These apparent K_ds do not necessarily reflect binding avidity in the cell, where solution conditions are not known.

Binding of Max/Max, truncated c-Myc/truncated c-Myc, and Myc/Max to free DNA and nucleosomal DNA. To determine whether dimer composition might affect the ability to bind to nucleosomal DNA, we first compared the abilities of Max and N-terminally truncated Myc, as either homo- or heterodimers, to bind to a CACGTG sequence that had been assembled into a nucleosome. These homo- and heterodimers all bind optimally to this sequence, allowing us to compare the abilities of different factors to bind to identical nucleosome preparations. All experiments were performed with c-Myc and Max proteins that were extensively purified from either bacterial or eukaryotic sources (see Materials and Methods).

By EMSA analysis, the nucleosome core particle migrates more slowly than free DNA (Fig. 1A, lanes 1 and 2). The addition of Max in increasing amounts to CACGTG-containing nucleosomes results in the appearance of a new slower mobility band, which is clearly distinct from the Max-free DNA complex (compare Fig. 1A, lanes 3 to 5). This band is not readily detectable when similar amounts of Max are incubated with nucleosomes lacking the CACGTG site (Fig. 1B, lane 4), implying that it is the result of specific binding. DNase I footprint titration confirms that Max binds specifically to the CACGTG site on nucleosomal DNA (Fig. 1D; note the concurrent 10-bp pattern of hypersensitive bands in lane 1 and the decreased cleavage of these bands over the binding site in lanes 5 and 6). Quantification of the decrease in intensity of these bands by phosphorimaging demonstrates that 90% of the nucleosomal DNA is bound specifically by Max. At higher concentrations of Max, however, additional regions of the DNA molecule are protected, compatible with nonspecific binding of Max adjacent to specifically bound Max (Fig. 1D, lane 7, and Fig. 1B). This provides a possible explanation for the more slowly migrating complexes in the EMSA (Fig. 1A, lanes 4 and 5, asterisk), which might represent nucleosomes to which two Max homodimers have bound, one specifically to the CACGTG site and the other nonspecifically. We do not know how many of the nonspecific sites on the nucleosome are represented as being bound in the upper complex. The affinity of any individual nonspecific interaction might, therefore, be similar to that for the specific complex (e.g., if only a small subset of the possible nonspecific sites are bound) or might be significantly weaker than that for the specific complex (e.g., if each of the approximately 140 nonspecific sites are represented equally in the upper complex).

The apparent K_ds for specific binding of Max homodimers to nucleosomal DNA (2.5×10^{-6} M) and free DNA (5.3×10^{-10} M) were calculated from the DNase footprint titration data (Fig. 1C and D and Table 1). Note that protein is in 100,000-fold molar excess to template at the midpoint of titrations that measure the apparent K_d for specific binding to nucleosomal DNA, so the amount of Max homodimers sequestered by nonspecific interactions should not significantly affect this calculation. We conclude that Max homodimers are able to bind specifically to nucleosomal DNA but that the affinity of this binding is weaker than for free DNA.

Purified full-length c-Myc (bacterially produced or baculovirus produced) binds extremely weakly to free DNA (30, 38; and data not shown). In the DNase footprint assay, only nonspecific interactions were detected for these full-length c-Myc proteins, precluding a determination of the apparent K_d for these homodimers. These full-length proteins are also

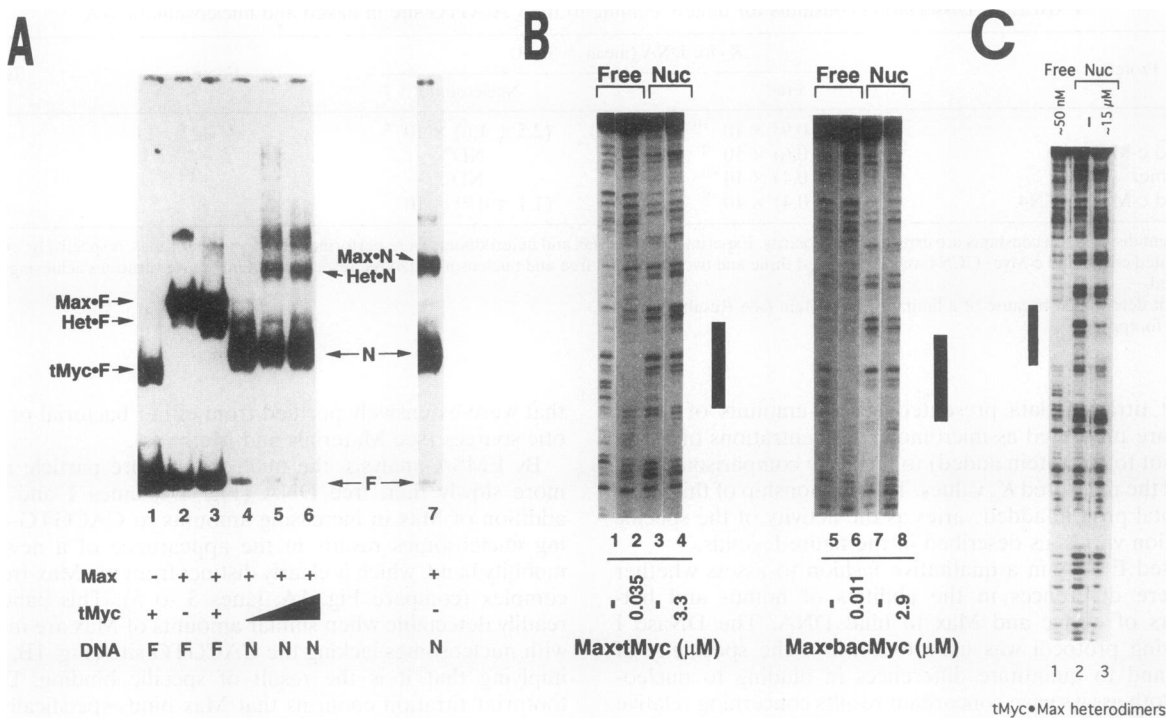

FIG. 3. Truncated c-Myc/Max heterodimers bind to both free DNA and nucleosomal DNA. (A) EMSA using labelled free (F) or nucleosomal (N, Nuc) DNA in the presence of the following amounts of bacterially produced purified Max and truncated c-Myc (tMyc) proteins: lane 1, 0 ng of Max and 25 ng of tMyc; lane 2, 1.5 ng of Max and 0 ng of tMyc; lane 3, 1.5 ng of Max and 3 ng of tMyc; lane 4, 1.5 ng of Max and 6 ng of tMyc; lane 5, 1.5 ng of Max and 12 ng of tMyc; lane 6, 1.5 ng of Max and 15 ng of tMyc; and lane 7, 4.5 ng of Max and 0 ng of tMyc. The mobilities of the homodimeric (tMyc · F, Max · F) and heterodimeric c-Myc/Max (Het · F) protein complexes with free DNA are indicated, as are the mobilities of Max (Max · N) and c-Myc/Max (Het · N) with nucleosomes. The Max · N complex migrates more slowly than the Het · N complex; although they were adjacent on the original gel, lanes 6 and 7 are separated for clarity (but see lanes 1 and 2 in Fig. 5B, which clearly demonstrate the distinct mobilities). The upper complexes in lanes 5 and 6 are nucleosomes with more than one dimer bound. (B) DNase I footprint with labelled nucleosomal DNA in the presence of the indicated concentrations of heterodimer (Max · tMyc or Max · full-length baculovirus c-Myc (bacMyc)). Max-tMyc heterodimer at 1 μM is equivalent to 3.2 μg of added protein. The position of the binding site and adjacent protected bases is indicated by the solid bar. (C) Binding of concentrated Myc/Max heterodimers to nucleosomal DNA. Concentrations of heterodimers are estimated from the extent of concentration and from the amount of heterodimer required to produce a footprint on small amounts of naked DNA. All lanes are from the same experiment.

unable to bind to nucleosomes by EMSA or DNase I footprinting (data not shown). A truncated c-Myc protein (30, 54) containing only the b-HLH-ZIP domains (c-Myc$^{342-439}$) is able to bind to free DNA, as measured by both EMSA and footprinting (Fig. 2A, lanes 2 and 3, and Fig. 2B). The apparent K_d of binding to free DNA was found to be comparable to that for Max (1.4×10^{-9} M) (Table 1). Truncated c-Myc, however, did not bind to nucleosomal DNA, as measured by either EMSA (Fig. 2A, lanes 4 to 7) or DNase I footprint titration (Fig. 2B). The amounts of truncated c-Myc used in the EMSA were significantly greater than those needed for Max to bind nucleosomal DNA. We conclude that although truncated c-Myc and Max homodimers bind free DNA equally well, c-Myc is less able to bind to the CACGTG site on a nucleosome than is Max.

c-Myc can form a heterodimer with Max. This heterodimerization has been demonstrated to dramatically increase the ability of full-length c-Myc to bind to free DNA (30). Given the difference in the abilities of c-Myc and Max homodimers to bind to nucleosomal DNA, we next examined nucleosome binding by c-Myc/Max heterodimers. These heterodimers bind with similar affinities to free DNA, regardless of whether truncated c-Myc, full-length bacterial c-Myc, or full-length phosphorylated baculovirus c-Myc is used (Table 1 and data not shown). Incubation of the truncated c-Myc/Max heterodimer with nucleosomes resulted in the appearance of several new bands in the EMSA (Fig. 3A, lanes 5 and 6). The lower band has a mobility that is different from that of the Max-nucleosome band (Fig. 3A, lane 7; also, see Fig. 5B to compare lanes 1 and 2), and the amount of Max that was used in this reaction mixture causes no demonstrable nucleosome supershift in the absence of c-Myc. The presence of bands with slower mobility suggests nonspecific binding of the heterodimer, similar to that seen with Max (described above). To determine whether binding was specific and to measure the apparent K_d for any specific interaction, we used DNase footprint titration. No specific DNase I footprinting on nucleosomes was demonstrated by the truncated or baculovirus c-Myc/Max heterodimeric complexes at a concentration of ≈3 μM c-Myc/Max (Fig. 3B). These data demonstrate that the apparent K_d for specific binding of c-Myc/Max to nucleosomes must be greater than 3 μM. DNase footprint assays were performed with heterodimer preparations that had been further concentrated by ultrafiltration. The yields of heterodimers after ultrafiltration were sufficiently poor that a titration to accurately determine the apparent K_d was not possible, although a partial specific footprint was observed (Fig. 3C). The simplest interpretation of these data is that, in spite of some

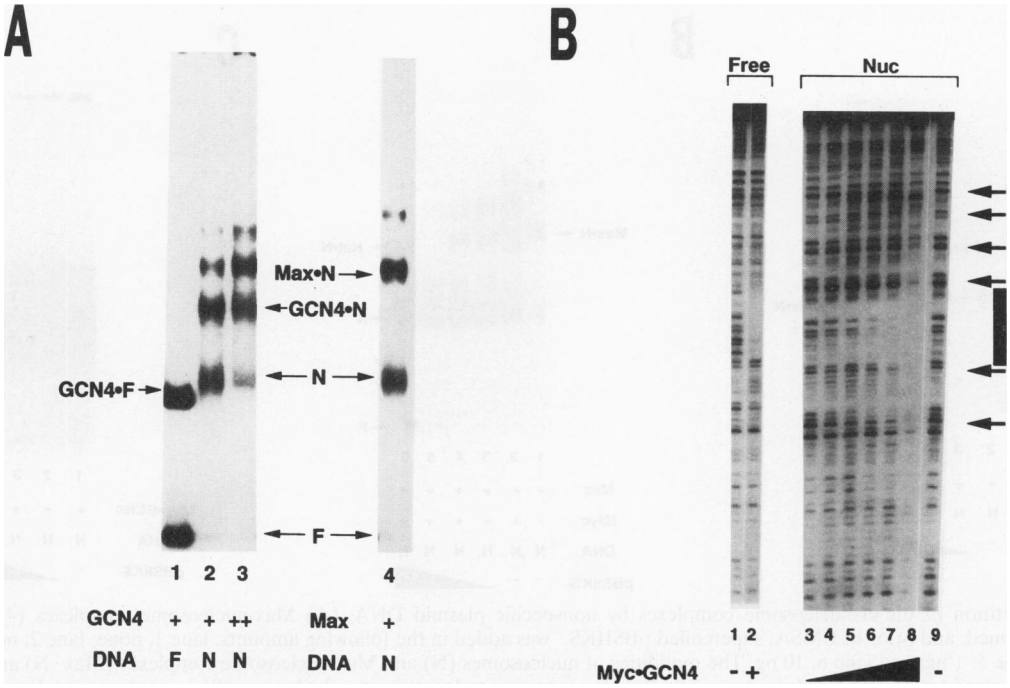

FIG. 4. Nucleosome binding properties of truncated c-Myc · GCN4. (A) EMSA using labelled free (F) or nucleosomal (N, Nuc) DNA in the presence of bacterially produced purified truncated c-Myc · GCN4 (GCN4) (lanes 1 and 2, 75 ng; and lane 3, 150 ng) or Max (lane 4, 4.5 ng) proteins. Complexes of GCN4 with free DNA (GCN4 · F) or with nucleosomal DNA (GCN4 · N) and of Max with nucleosomal DNA (Max · N) are indicated. The slower-mobility bands in lanes 2 to 4 represent nucleosomes with more than one bound dimer. All samples were run on the same gel, but lanes 3 and 4 were separated for purposes of clarity. (B) DNase I footprint with labelled free and nucleosomal DNA and the following concentrations of GCN4 (in micromolar concentration): lanes 1 and 9, 0; lane 2, 0.004; lane 3, 0.013; lane 4, 0.043; lane 5, 0.129; lane 6, 0.43; lane 7, 1.29; and lane 8, 4.17. Active truncated Myc-GCN4 at 1 μM is equivalent to 24 μg of total protein in a 100-μl reaction. The position of the binding site and adjacent protected bases is indicated by the solid bar, and the arrows show sites of enhanced cleavage in nucleosomal DNA separated by areas of increased protection.

weak nonspecific heterodimer binding, the truncated c-Myc/Max heterodimers bind specifically (as evidenced by the EMSA band and the presence of a footprint) but weakly ($K_d > 3 \times 10^{-6}$ M) to nucleosomes.

Truncated c-Myc · GCN4 binds both free and nucleosomal DNAs. The above data demonstrate that although Max homodimers, truncated c-Myc homodimers and c-Myc/Max heterodimers all bind free DNA with similar affinities, they differ in their abilities to bind to nucleosomal DNA. All of these proteins bind to the same sequence, and all proteins were tested for binding to identical nucleosome preparations, so the positions of the binding site on the nucleosome were identical. These data therefore suggest that the difference in ability to bind to nucleosomes by these homo- and heterodimers was caused by some functional difference between these related dimers: it appeared that an aspect of the structure of Max homodimers differed from that of the truncated Myc homodimers in a manner that specifically affected nucleosomal binding. We sought to confirm and extend this hypothesis by determining whether we could increase the ability of truncated c-Myc homodimers to bind to nucleosomal DNA by mutating the truncated c-Myc protein. c-Myc and Max have similar basic regions, as expected because they bind the same sequence, but are known to differ in the abilities of their dimerization domains to function (c-Myc homodimerizes less effectively than Max). We therefore altered the dimerization domain of truncated c-Myc to determine whether this change would alter nucleosomal binding.

c-Myc is a member of the b-HLH-ZIP class of proteins, and it has been demonstrated that the ZIP portion of the dimerization domain can play a critical role in dimer stability. To determine whether altering the dimerization domain could affect nucleosomal binding, we created a truncated c-Myc protein in which the native ZIP was replaced with that of the yeast transcription factor GCN4 (we initially attempted to replace the dimerization domain of Myc with that of Max, but were unable to produce this protein). The GCN4 ZIP is known to homodimerize efficiently and should promote more stable dimerization. Truncated c-Myc · GCN4 and truncated c-Myc therefore share identical basic (DNA binding) and HLH motif and differ only in the ZIP region. Both proteins had similar electrophoretic mobilities by SDS-PAGE (data not shown).

Truncated c-Myc · GCN4 protein binds to free DNA (Fig. 4A, lane 1); the apparent K_d of this binding is the same as that for truncated c-Myc (Table 1). In contrast to truncated c-Myc, truncated c-Myc · GCN4 dimers bind to nucleosomal DNA (Fig. 4A, lanes 2 and 3). The mobility of the truncated c-Myc · GCN4-nucleosome complex is distinct from that of the Max-nucleosome complex (Fig. 4A, lane 4), as are the respective complexes containing free DNA. The binding of truncated c-Myc · GCN4 to nucleosomal DNA is specific (Fig. 4B, compare lanes 3, 6, and 7), and the affinity, as measured by DNase footprint titration, is significantly greater than that of truncated c-Myc for the same template (Fig. 4B and Table 1) and is approximately 10-fold greater than Max binding to a nucleosome. As with Max binding to nucleosomes, we also observed evidence for nonspecific binding adjacent to the specific sites, indicated by additional complexes of slower mobility on EMSA

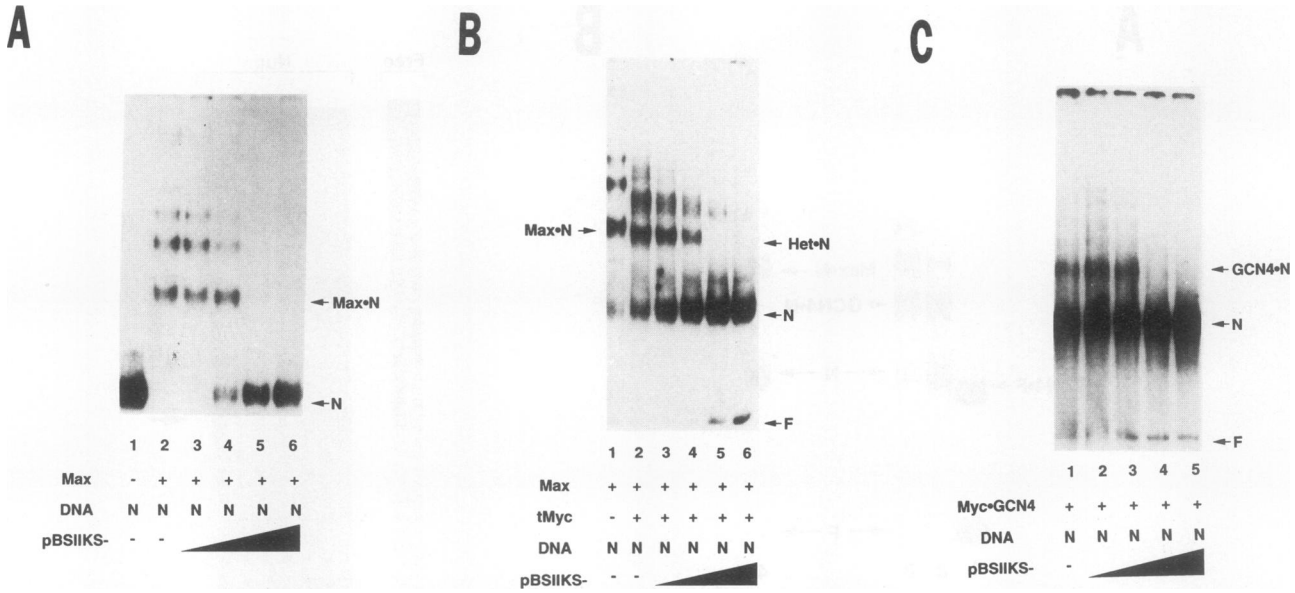

FIG. 5. Competition of dimer-nucleosome complexes by nonspecific plasmid DNA. (A) Max-nucleosome complexes (4.5 ng of Max per reaction) were formed, and prior to EMSA, supercoiled pBSIIKS$^-$ was added in the following amounts: lane 1, none; lane 2, none; lane 3, 10 pg; lane 4, 100 pg; lane 5, 1 ng; and lane 6, 10 ng. The mobilities of nucleosomes (N) and Max-nucleosome complexes (Max · N) are indicated. As in Fig. 1A, the more slowly migrating bands in lanes 2 to 4 likely represent nucleosomes to which more than one dimer has bound nonspecifically. The gel from which this autoradiograph was obtained was electrophoresed until the free DNA had migrated off the gel and is therefore not shown in its entirety. (B) Truncated c-Myc (tMyc)Max-nucleosome complexes were formed (1.5 ng of Max and 15 ng of truncated c-Myc per reaction) and competition was carried out as described above, with the following amounts of pBSIIKS$^-$: Lane 1, none; lane 2, none; lane 3, 10 pg; lane 4, 100 pg; lane 5, 1 ng; lane 6, 10 ng. (C) Truncated c-Myc · GCN4-nucleosome complexes were formed, and prior to EMSA, pBSIIKS$^-$ was added in the following amounts: lane 1, none; lane 2, none; lane 3, 10 pg; lane 4, 100 pg; lane 5, 1 ng; and lane 6, 10 ng. The mobilities of nucleosomes (N) and GCN4-nucleosome complexes (GCN4 · N) are indicated. As in previous figures, the more slowly migrating bands in lanes 2 to 4 likely represent nucleosomes to which more than one dimer has bound nonspecifically.

(Fig. 4A, lane 3), and adjacent protected regions at high concentrations in the DNase footprint titration (Fig. 4B, lane 8). We conclude that changes in the dimerization domain that have no effect on binding to free DNA can dramatically alter binding to nucleosomal DNA.

Binding of Max, truncated c-Myc/Max or truncated c-Myc · GCN4 does not destabilize the nucleosome. Binding to nucleosomal DNA by any of these proteins resulted in a complex that migrated more slowly in EMSA than the protein-free DNA complex. This suggested the possibility that the intact nucleosome was still present in these complexes, as was also suggested by the continued 10-bp periodicity, characteristic of nucleosomal DNA, that surrounded the footprints of Max and truncated c-Myc · GCN4. It has been shown that binding of GAL4 protein to multiple sites destabilized the underlying nucleosome (56). We therefore determined whether an intact nucleosome was still present in these complexes and tested the stability of the underlying nucleosome.

Protein-nucleosome complexes were formed, and increasing amounts of a supercoiled plasmid lacking the CACGTG sequence were added to the reaction mixture. If binding of Max/Max homodimers, truncated c-Myc/Max heterodimers, or truncated c-Myc · GCN4 homodimers destabilizes the nucleosome, complete histone dissociation should produce EMSA bands with the mobility of the respective dimers on free DNA. Alternatively, if the nucleosome remains intact and stable, dissociation of the dimer should yield a free nucleosome band. If histones are still present but in an altered conformation, new EMSA bands with distinct mobilities might appear. For Max homodimers, addition of 1 ng of nonspecific plasmid resulted in the reappearance of the original nucleosome band. The concomitant increase in intensity of the nucleosome band and decrease in intensity of the Max-nucleosome band (Fig. 5A) suggest that binding of Max does not destabilize the nucleosome. Identical results were observed with the truncated c-Myc/Max heterodimer (Fig. 5B) and the truncated c-Myc · GCN4 homodimer (Fig. 5C). These data demonstrate that the nucleosome in these bound complexes remained stable.

DISCUSSION

Several lines of evidence argue that binding to nucleosomal DNA and the subsequent destabilization of the surrounding chromatin structure by transcriptional activators is likely to play an important role in gene regulation (reviewed in references 3, 18, 24, 26, and 49 to 51). Many activators bind DNA as homo- or heterodimers, and changes of dimerization partner are believed to have important regulatory consequences for members of the b-ZIP, b-HLH and b-HLH-ZIP classes. We propose that changes of dimerization partner will have significant effects on the ability of activators to bind to nucleosomal DNA and that these effects will not necessarily be reflected in changes in the ability to bind to free DNA. We have demonstrated that changes of dimerization partner alter the abilities of c-Myc and Max to interact with nucleosomal templates.

Transcription factors that have obvious differences in DNA binding domain structure have been shown to bind to nucleosomal DNA with affinities that are not predicted from free DNA binding affinity. The glucocorticoid receptor and NF1 differ in their abilities to bind a nucleosome in the mouse mammary tumor virus enhancer (4, 41). GAL4 binds nucleosomal DNA more avidly than HSF, despite the ability of HSF

to bind free DNA 2 orders of magnitude more avidly than GAL4 (48). These differences in nucleosomal binding might reflect differences in rotational or translational position of the site on a nucleosome, might reflect the structure of the DNA binding domain, or most likely, might reflect a combination of these effects. The relative contributions of DNA binding domain structure and of binding site position have been difficult to examine with different transcription factors because of the need to compare binding to sites at all 10 possible rotational positions as well as different translational positions. We have taken a different approach by analyzing binding at a single position on the nucleosome and altering the transcription factor. We have rigorously demonstrated that the composition of the DNA binding and dimerization domains plays a necessary role in determining the ability of b-HLH-ZIP factors to bind to nucleosomal DNA. For example, replacing the c-Myc ZIP with the GCN4 ZIP increased binding to a specifically positioned site on a nucleosome by more than an order of magnitude (Table 1). These dimers, as well as all of the other combinations of c-Myc and Max that we examined, bound free DNA equivalently.

Whether these differences in nucleosomal binding reflect increased dimer stability as opposed to an alteration in the orientation of the DNA binding region is unclear. It is tempting to speculate that tightening of the ZIP interaction could modify the alignment of the DNA binding regions with respect to each other. On the basis of earlier models of b-HLH-ZIP proteins (16, 23, 52) and the recently described Max crystal structure (19), a tighter ZIP interaction might restrict dynamic pivoting of the monomers about an axis perpendicular to the extended coiled coil formed by helix 2 and the ZIP, modifying the orientation of the basic regions relative to one another. Previous studies have indicated that alterations within the basic region can modify the specificity of DNA recognition (15); the data presented here demonstrate that changes outside the DNA binding region per se are able to modify the quality of DNA binding.

The ability of a transcription factor to induce a directed bend in the DNA (31, 54) that is compatible with nucleosomal structure might be important to nucleosomal binding. c-Myc and Max bend free DNA in diametrically opposite orientations, with Max homodimers bending DNA away from the protein and truncated c-Myc homodimers bending DNA toward the protein; bending by c-Myc/Max heterodimers is also directed away from the protein, but is of a smaller magnitude (54). These findings have been recently confirmed and extended to other members of the HLH-ZIP family (20). Surprisingly, the recently described three-dimensional crystal structure of Max demonstrates no DNA bending (19). As the authors of that study point out, however, this discrepancy with the solution studies might result from crystal packing effects.

Because DNA in the nucleosome is wrapped around the histone core, and therefore inherently bent, a transcription factor which prefers this bent DNA conformation might preferentially allow specific base recognition in nucleosomal DNA. The orientation of DNA bending observed with free DNA might indicate such a preference, and this property might be inherent to a given pair of basic regions and, through dimerization, their particular alignment (i.e., DNA "bending" might result from passive deformation of free DNA as it fits into the DNA binding domain) (54). Based on the orientation of DNA bending observed, this model predicts that bending away from the protein would be expected to favor nucleosome binding. Thus, Max homodimers and c-Myc/Max heterodimers should be able to bind nucleosomal DNA, but truncated c-Myc homodimers should show no nucleosome binding. This hypothesis is consistent with our results (Table 1). The pattern of DNA bending exhibited by truncated c-Myc · GCN4 was clearly different from that of truncated c-Myc, in spite of the fact that they share both basic and HLH domains; truncated c-Myc · GCN4 was similar to Max/Max and c-Myc/Max in that bending was oriented away from the protein (data not shown). Thus, nucleosome binding correlates with the orientation of DNA bending in the examples studied here. A more exhaustive analysis is needed to determine if this model is generally applicable.

Nucleosomes are believed to inhibit transcription by physically blocking access of both activators and general transcription factors (18, 21, 35, 36, 55, 57). Thus, in order for transcription to occur, histones must at least temporarily alter their association with the DNA (3, 12, 32, 33, 49, 51). We examined whether c-Myc or Max could destabilize a nucleosome directly, as has been shown to occur when GAL4 binds tandem sites on a nucleosome (56). The addition of excess nonspecific DNA caused Max/Max, truncated c-Myc/Max and truncated c-Myc · GCN4 homodimers to dissociate from the nucleosome, without apparently disrupting the nucleosome-DNA interaction. This suggests that neither the homodimeric nor the heterodimeric complexes alone are likely to relieve nucleosome-mediated transcriptional repression by directly liberating DNA from histones. This in vitro study, however, does not preclude the possibility that c-Myc or Max might interact with other proteins to effect changes in chromatin architecture in vivo. Furthermore, the fact that the truncated c-Myc/Max heterodimer does not dissociate nucleosomes does not necessarily mean that the full-length c-Myc/Max heterodimer would be unable to do so in the context of a natural promoter in vivo. Unfortunately, the inherently poor activity of purified full-length c-Myc protein made it impossible to detect any nucleosome binding by EMSA.

It is not clear how to interpret the above data in terms of the ability of these proteins to bind to chromatin in vivo. The apparent K_ds (Table 1) were determined under a single set of solution conditions, and it is not known how these conditions relate to conditions in the nucleus. In addition, it is not clear how to relate binding constants measured in vitro to the function of a factor in an intact cell. It is possible, for example, that a low level of binding by a factor would allow a binding site to be occupied transiently, but for a period sufficient to recruit other factors that might destabilize the underlying nucleosome. Thus, in vivo, it is possible that a weak interaction of Max with its binding site on a nucleosome might trigger a destabilization of the nucleosome to allow a transition to a more stable (i.e., lower apparent K_d) binding state. At present, there are no data to support this hypothesis; we mention it to illustrate the difficulties in predicting the in vivo behavior of these factors in binding to chromatin from measurements made in vitro. A final possibility is that function of these proteins in vivo might be influenced by other factors that alter the chromatin structure surrounding the CACGTG binding site. A direct test of this hypothesis depends on the characterization of the chromatin structure and factor binding sites in an enhancer or promoter region that surround a site known to bind Max or c-Myc/Max in vivo; such a system does not presently exist.

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D.S.W. and O.P. contributed equally to this work.

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