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# Molecular characterization of the GCN4–DNA complex

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**ABSTRACT** **We report studies of the DNA complex formed by GCN4, a transcriptional activator of eukaryotic amino acid biosynthetic operons. The DNA thermodynamic binding domain, defined by primer extension analysis, spans at least 18 base pairs, a site much larger than the 9-base-pair consensus defined by homology with naturally occurring binding sites. Chemical modification experiments reveal multiple sites of protein–DNA contact: methylation of any guanine N-7 or adenine N-3, ethylation of any phosphate oxygen, or elimination of any nucleoside within a region spanning nearly one and a half turns of the double helix reduces the binding affinity of the complex measurably. Nevertheless, the protein yields no detectable hydroxyl radical footprint, implying that the minor groove is reagent-accessible in the protein–DNA complex. These chemical modification patterns indicate that GCN4 does not utilize any of the DNA-recognition motifs of paradigm DNA-binding proteins. Assays to detect DNA bending induced by truncated or intact GCN4 indicate that protein conformation and not a protein-induced bend is responsible for the anomalous electrophoretic behavior of GCN4–DNA complexes.**

In *Saccharomyces cerevisiae* a general control system coordinately regulates the expression of unlinked genes encoding amino acid biosynthetic enzymes (1). Genetic and biochemical studies (2–6) have shown that the regulatory protein GCN4 binds to upstream TGACTC sequences and activates transcription in response to amino acid starvation. GCN4 is a modular protein with an internal 32-amino acid transcriptional activation domain, rich in acidic residues (7, 8), and a C-terminal 60-amino acid DNA-binding and protein-dimerization domain (7, 9, 10). The latter contains leucines spaced exactly 7 amino acids apart, a characteristic feature of a motif termed the "leucine zipper" (11) that appears to be involved in the dimerization of C/EBP (12) and the heterodimerization of JUN oncoprotein with FOS (13–17). A 25-residue region immediately adjacent to the N-terminal end of the dimerization domain is highly conserved between GCN4 and JUN (18); the two proteins bind to similar DNA sites (19) and it is likely that this region, rich in basic residues, is involved in sequence-specific protein–DNA contacts. The amino acid sequence of the basic domain is not related to the sequence of any of the paradigm DNA-recognition structures such as the zinc "finger" or helix–turn–helix.

Extensive mutational analysis based on oligonucleotide-directed sequence randomization and affinity chromatography has revealed that the recognition site spans 13 base pairs (bp) (20), a region significantly larger than the 9-bp consensus defined by homology with naturally occurring GCN4 binding sites (21). The central 7 bp are most critical for tight association (20, 21). The experiments reported here serve to identify the DNA domain that contributes to the full equilibrium stability of the complex, and to elucidate the nature of protein–DNA contacts within the domain. We have also examined the ability of GCN4 to induce DNA bending. The pattern of interaction between GCN4 and DNA appears to be unrelated to any well-characterized protein–DNA complex.

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## MATERIALS AND METHODS

**DNA Constructs and Fragments.** Primers and templates for the primer extension analysis were synthesized on an Applied Biosystems oligonucleotide synthesizer by the phosphoramidite method and were purified by preparative gel electrophoresis. For chemical modification experiments, an oligonucleotide carrying a GCN4 super site, *his3-189* (see ref. 20), was cloned into the polylinker of a pGEM-2 (Promega) derivative carrying a *lac* promoter fragment (22). The resulting plasmid (pGCNCAP) was constructed from the following sequences:

```
5'-GATCTGGATGACTCATTTTTTTTTGCTCGAGCTTCTATGAGGCCTGAGCT-3'
    3'-ACCTACTGAGTAAAAAAAAACGAGCTCGAAGATACTCCGGAC-5'
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Singly 3'-end-labeled fragments for chemical modification were generated with the Klenow fragment of DNA polymerase I (New England Biolabs), [$\alpha$-$^{32}$P]dNTPs (Amersham), and selective enzymatic cleavage.

A 195-bp binding-site fragment from a pGCNCAP derivative was isolated and cloned as a tandem dimer for the circular permutation assay. The fragment contained four unique restriction sites: *Ava* I, *Bam*HI, *Eco*RI, and *Msp* I. Plasmids containing four phased adenine tracts were generously provided by Tali Haran (Yale University). Two-hundred-base-pair fragments containing the adenine tracts and variable spacer were isolated and cloned into pGCNCAP adjacent to the GCN4 binding sites; 300-bp fragments with both loci $\approx$100 bp from their respective molecular ends were isolated.

**Proteins and Enzymes.** *Escherichia coli* strain LC137, plasmid pAB100 (6), and plasmid pCJ136 were gifts of A. Goldberg (Harvard University), G. R. Fink (Massachusetts Institute of Technology), and C. Joyce (Yale University), respectively. LC137 was cotransformed with pAB100, which harbors *GCN4* under the control of a $\lambda$ $P_L$ promoter, and pCJ136, which harbors the gene for a temperature-sensitive $\lambda$ cI repressor. Induced cultures were lysed with a French pressure cell in 200 mM NaCl/25 mM Hepes, pH 7.0/0.2 mM EDTA/0.2 mM phenylmethylsulfonyl fluoride/0.5 mM dithiothreitol. Cleared lysates were treated with 0.5% Polymin-P (BRL), the resulting precipitate was removed by centrifugation, and GCN4 was precipitated from the supernatant with 25%-saturated ammonium sulfate. The pellet was redissolved in lysis buffer (without phenylmethylsulfonyl fluoride and dithiothreitol) and purified by cation-exchange chromatography (Mono S; Pharmacia). GCN4-containing fractions were pooled, dialyzed against 100 mM NaCl/10 mM Tris·HCl/0.2 mM EDTA, pH 8.0, and purified further by anion-exchange chromatography (Mono Q; Pharmacia). The protocol yields GCN4 of suitable purity for immediate DNA binding assays; however, a minor protease activity copurifies with the protein and over a period of several weeks, truncated GCN4 derivatives accumulate to significant levels. A 65-amino acid C-terminal proteolytic fragment containing the DNA binding

and protein dimerization activity, termed δGCN4, was partially purified from the degradation mixture by additional anion-exchange chromatography (Mono Q). All other enzymes were purchased (New England Biolabs).

**Gel Electrophoresis.** Complexes of GCN4 with DNA fragments larger than 100 bp were separated by electrophoresis in 5% polyacrylamide gels (75:1 acrylamide/*N,N'*-methylenebisacrylamide weight ratio); 10% gels were used for complexes with DNA fragments 70 bp and shorter. Gels were electrophoresed in 0.5× TBE buffer (45 mM Tris/45 mM boric acid/1 mM EDTA, pH 8.3) at 420 V in a constant-temperature gel apparatus (Hoefer) at 25°C. Denaturing 10%, 12%, and 14% gels (7 M urea, 19:1 acrylamide/*N,N'*-methylenebisacrylamide, 1× TBE) were used to separate DNAs to nucleotide resolution for the chemical modification, primer extension, and oligonucleotide competitive binding experiments, respectively.

**Primer Extension.** The experimental protocol was based on the method of Liu-Johnson *et al.* (23) with the following modifications: 5'-end-labeled oligonucleotides were purified from contaminating free label by Sephadex G-10 spin chromatography. For each base-specific primer extension reaction the competing dNTP was 0.83 μM and the other remaining dNTPs were 33 μM. The ratios of the dideoxynucleoside triphosphate to the competing dNTP were 750:1 for adenine, 600:1 for thymine, 340:1 for cytosine, and 170:1 for guanine. Base-specific primer extension reactions were terminated by the addition of phenol/chloroform. Subsequently, they were pooled, chloroform-extracted, dried, and resuspended in TE buffer (10 mM Tris·HCl/1 mM EDTA, pH 8.0). All complexes were formed in 100 mM NaCl/3 mM $MgCl_2$/20 mM Tris·HCl, pH 7.4. The reaction mixture was incubated for at least 10 min to ensure equilibrium before gel loading. Bands containing bound and free DNAs were excised and eluted into 40 mM NaCl in TE. Band intensities were measured with a Betagen blot analyzer.

Template strands from the primer extension reactions were annealed to yield the duplex 50-mer used in the oligonucleotide competitive binding experiment. The sequences of the complementary 19-bp oligonucleotides also used were CGGATGACTCTTTTTTTTT and AAAAAAAAAGAGTCATCCG. The electrophoretically resolved band corresponding to bound 19- and 50-mer was excised and 100 mM Tris·HCl, pH 7.8/50 mM EDTA/5% SDS containing proteinase K at 50 μg/ml was applied directly to the surface of the gel slice. After 20 min at 37°C, the gel slice was sandwiched between glass plates and a denaturing gel was poured around it (see *Gel Electrophoresis*).

**Chemical Modification.** The nucleoside elimination (24) protocol was based on the hydroxyl radical footprinting procedure (25) with the exception that protein was omitted. Modified DNA was ethanol-precipitated, resuspended in TE, and bound by protein in 100 mM NaCl/3 mM $MgCl_2$/20 mM Tris·HCl, pH 7.4. Alkylation protocols were based entirely on the methods of Hendrickson and Shleif (26). Band intensities from the chemical modification experiments were measured by densitometry.

## RESULTS AND DISCUSSION

**Mapping the Thermodynamic Binding Domain.** In the primer extension method of Liu-Johnson *et al.* (23), one quantitates the relative binding affinities of DNA fragments differing successively by a single base pair in duplex length. Fragments containing part or all of the GCN4 binding site were generated by Klenow fragment-catalyzed primer extension, in the presence of dideoxynucleoside triphosphates. Sequences for the primers and templates, shown below, were derived from the *S. cerevisiae HIS3* promoter, where an asterisk marks the binding-site pseudo-dyad axis.

```
5'-CGTTTTTTTTCCACC-3'

3'-GCAAAAAAAAGGTGGATCGCCTACTGAGAAAAAAAAAAGAATCGCTAACCG-5'
                            *
                            *
5'-CGTTTTTTTTCCACCTAGCGGATGACTCTTTTTTTTTTCTTAGCGATTGGC-3'

                                    3'-GAATCGCTAACCG-5'
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To ensure discrimination, a limiting quantity of GCN4 was equilibrated with the set of DNA molecules. The free and bound DNA products of the partition reaction were resolved by nondenaturing gel electrophoresis, eluted, and resolved by electrophoresis through sequencing gels. Though all fragment lengths were represented in the unbound lane, only fragments with a sufficient portion of the GCN4 thermodynamic binding domain appeared in the bound lane. The ratio ($K_n$) of protein-bound DNA ($C_n$) and free DNA ($D_n$) for each length $n$ was determined and the relative binding affinity $K_{rel}(n)$ was calculated from the ratio $K_n/K_{full}$, where $K_{full}$ represents the ratio of bound and free DNA of sufficient length so that binding is independent of length.

Fig. 1 shows plots of relative binding affinity versus the position at which primer extension terminates, demonstrating that the thermodynamic binding domain for GCN4 in the *HIS3* promoter spans at least 18 bp. The relative binding affinity increases sharply as the primer is extended from the central G·C pair to a point 6 bp beyond the binding-site pseudo-dyad axis in a direction away from the transcription start, after which there is no detectable systematic effect of duplex length on binding affinity (Fig. 1 *Lower*). The binding constant rises more gradually as the primer is extended in the other direction, up to 11 bp beyond the pseudo-dyad axis toward the transcription start (Fig. 1 *Upper*). It is probably no coincidence that the gradual increase in binding free energy on the promoter-proximal side of the binding site corresponds to the addition of 9 contiguous thymines. Both

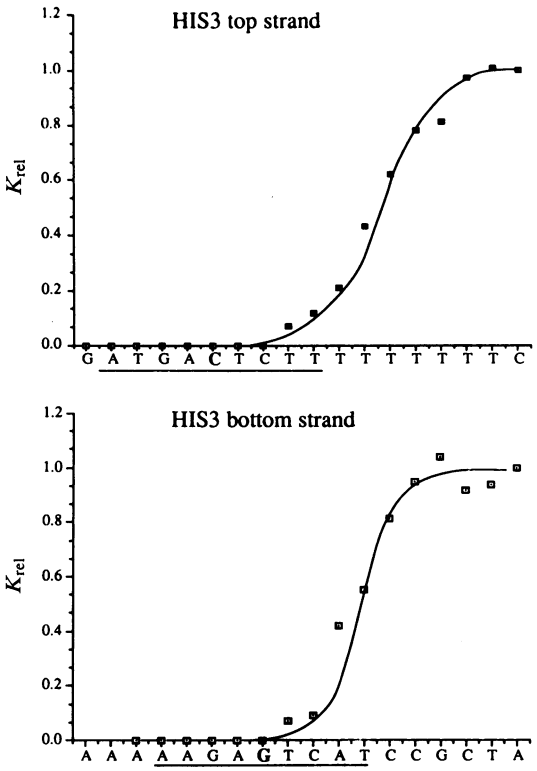


FIG. 1. Primer extension mapping of the thermodynamic binding domain. The plots show relative binding affinity as a function of binding-fragment duplex length. The 9-bp consensus domain is underlined.

NMR and gel electrophoresis studies of adenine-tract-containing oligonucleotides reveal substantial structural changes as the length of the adenine tract increases, particularly within a window of 3–7 adenosines (27–29). An altered DNA structure within the *HIS3* promoter adenine-tract region may potentiate GCN4 binding, even if only the portion of the adenine tract nearest the binding site center contacts the protein.

Other lines of evidence support the involvement of the adenine tract in GCN4 binding. Foremost, GCN4 enhances DNase I digestion at the distal end of the tract, 11 bp from the binding-site dyad axis (5). In addition, sequence alterations in the 4 proximal adenines of the tract reduce GCN4 affinity *in vitro* and transcriptional initiation *in vivo* (20). Extensive thermodynamic DNA binding domains such as those observed for GCN4 and *E. coli* catabolite gene activator protein (23) increase the information content used to specify the structure and stability of the cognate protein–DNA complexes, features necessary for these proteins to attenuate the induction of multiple operons.

The DNA fragments generated by primer extension differ in duplex length but share a common residual single-stranded template. The possible contribution of extra DNA flanking the binding site to the binding affinity was examined by comparing the relative binding affinity of a duplex 19- and 50-mer, both harboring the full thermodynamic binding domain; the method used was analogous to the primer extension experiments. We found that the 19-mer binds half as well as the 50-mer, from which we infer that the single-stranded tails of primer-extended fragments probably contribute minimally to the apparent relative binding affinity.

**Influence of Missing Contacts on Protein Binding Affinity.** DNA contacts essential for protein binding were identified by a nucleoside-elimination procedure (24). DNA containing the full thermodynamic binding domain was treated with iron/EDTA-generated hydroxyl radicals, which promote oxidative degradation of the deoxyribose ring and produce a small fractional population of gapped duplexes (30). Limiting GCN4 was equilibrated with the set of partially modified DNAs and the products were separated in nondenaturing gels and resolved by electrophoresis through sequencing gels. Gaps at 12 contiguous nucleosides of the top strand and 14 contiguous nucleosides of the bottom strand were found to be preferentially excluded from the protein–DNA complex. Fig. 2 plots the reduction in relative binding free energy corresponding to the loss of each contacted nucleoside. The size of the binding site defined this way is smaller than the thermodynamically defined binding domain. Most of the difference lies in the A·T base pairs at the distal end of the adenine tract. As discussed above, the entire adenine tract may mediate protein binding affinity through the formation of an alternative conformation, even if only a portion of the tract is contacted by the protein.

The analogous hydroxyl radical footprinting experiment provides no discernible protein protection pattern (data not shown). This result is not due to protein dissociation during the course of the experiment, since GCN4-dependent band-shifts were obtained before and after the reaction. Hydroxyl radicals are believed to abstract the 4′ sugar proton (31), which lies in the minor groove of canonical B-form DNA. Evidently, the minor groove of the binding site is exposed to small reactive molecules when bound by GCN4. In apparent contradiction, the protein partially protects a 10-bp region from digestion by DNase I (5), a bulky enzymatic probe of minor-groove accessibility and distortion. Unlike hydroxyl radical footprinting, inhibition of DNase I cutting may result from steric hindrance provided by a portion of GCN4 that does not contact the DNA directly or from protein-induced DNA distortion.

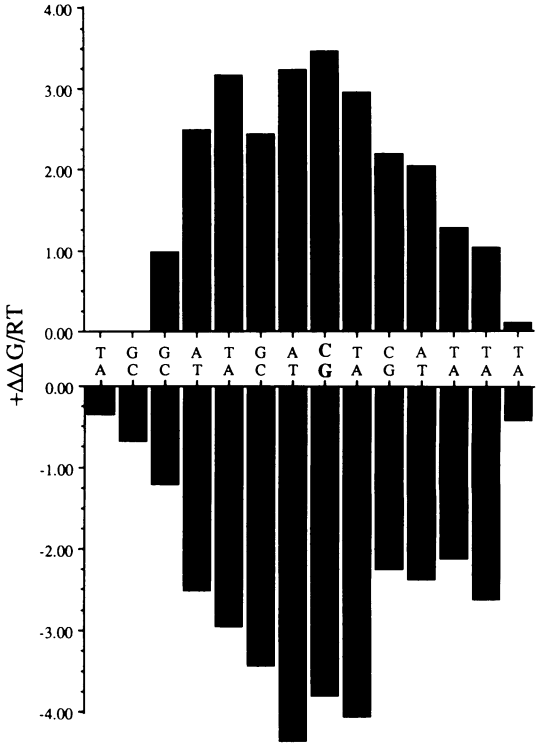


FIG. 2. Influence of missing DNA contacts on GCN4 binding, showing the reduction in relative binding Gibbs free energy (kcal/mol) as a function of eliminated nucleoside position. The central base pair is shown in larger, boldface type.

**Interference with Protein Binding by Nucleotide Alkylation.** Naked duplex DNA was mildly alkylated with ethylnitrosourea or dimethyl sulfate to identify functional groups within the binding site that intimately contact the protein (26, 32); ethylnitrosourea ethylates the oxygens of phosphates, and dimethyl sulfate methylates the N-7 of guanine and the N-3 of adenine. Partially modified DNA was incubated with limiting GCN4 and electrophoresed through nondenaturing gels. Protein-bound and free DNAs were eluted, treated with NaOH to cleave modified nucleotides, and resolved in sequencing gels. Ethylation of any phosphate oxygen in regions spanning roughly 11 nucleotides of both strands interferes with protein binding (Fig. 3). The 3-bp 5′ stagger of the interference pattern is indicative of steric hindrance of major-groove accessibility; in three dimensions, the phosphates of opposite strands that approach each other most closely are displaced by 2–3 bp to the 5′ side when spanning the major groove and by 2–3 bp to the 3′ side when spanning the minor groove.

Protein binding is inhibited by methylation of any adenine or guanine within the sequence 5′-GATGA$\overset{*}{C}$TCA-3′ on the upper strand and 5′-AAATGA$\overset{*}{G}$TCA-3′ on the lower strand (the asterisks indicate the central C·G base pair). Since adenine N-3 is in the minor groove and guanine N-7 is in the major groove, the interference pattern implies simultaneous protein contact of both major and minor grooves of the binding site, a result in apparent conflict with the inability to observe a hydroxyl radical footprint and with the 5′-staggered ethylation interference pattern described above. Contradictions of this nature may reflect the limitations of chemical interference techniques; inhibition of protein binding by alkylation cannot always be attributed to the *direct* loss of essential contacts because chemical modification simultaneously alters the electronic and structural properties of the DNA double helix. The absence of hydroxyl radical protection indicates that the minor groove is reagent-accessible;

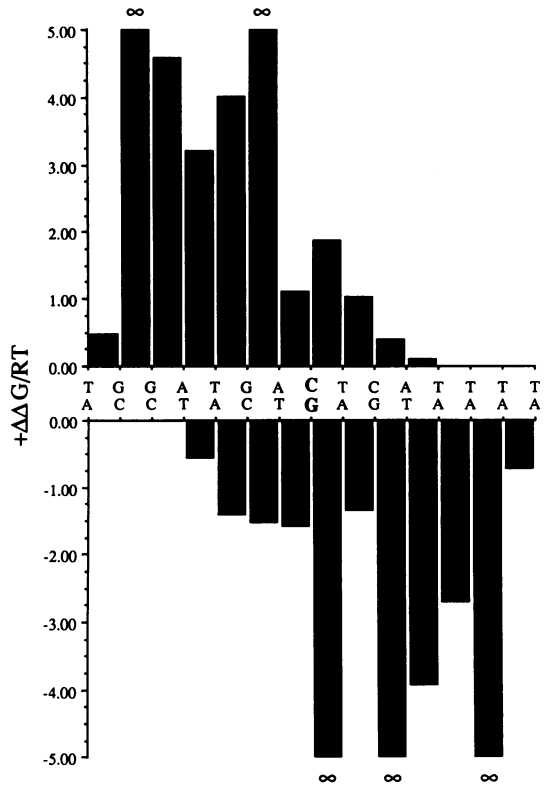


FIG. 3. Plot of the reduction in relative binding Gibbs free energy (kcal/mol) as a function of ethylated phosphate position. The ∞ symbol indicates that the value is very large but undetermined with the present data.

inhibition of protein binding by methylation in the minor groove may result from transmission of an electronic or structural perturbation of the DNA that affects recognition features that lie in the major groove. In addition, some chemical damage events may act by interfering with a DNA distortion necessary for binding (33).

With the possible exception of *Eco*RI (34, 35), the alkylation interference patterns of paradigm site-specific DNA-binding proteins provide no clear precedents for the GCN4 data, suggesting that the protein may use a novel recognition motif to bind DNA. Recently, the enhancer-binding protein EBP1 was subjected to similar analyses and found to produce related but distinguishable interference patterns: EBP1 makes base and backbone contacts over 12 contiguous base pairs (36). Moreover, chemical modification data for two other leucine-repeat proteins, C/EBP and chimeric FOS homodimers, parallel our results (37, 38).

**Protein-Induced DNA Bending.** The circular permutation assay (39) was employed to detect protein-induced DNA bending. Protein-bound circularly permuted 195-bp fragments were electrophoresed in nondenaturing gels (Fig. 4*a*); the *Eco*RI fragment, which positions the recognition sequence closest to the molecular center, is most retarded by GCN4 binding, as expected if the protein induces a bend in its binding site. However, the position-dependent variation in mobility induced by GCN4 is relatively small and may, as we discuss below, arise from sources other than bending.

To determine the relative direction of the apparent protein-induced bend, a fragment containing a GCN4 binding site was fused to a fragment containing phased adenine tracts whose bending direction and magnitude have been elucidated (40, 41). The spacing between the two loci was varied over a full turn of the double helix; if GCN4 bends DNA, as suggested by the previous assay, the set of protein-bound spacer constructs should exhibit variations in gel mobility that correspond to the relative bend directions and phasing of the bend centers with the helical repeat of the intervening DNA. Fig. 4*b* reveals that the mobilities of all protein-bound constructs are nearly invariant (although the free DNAs display detectably different mobilities due to the amplification of inherent DNA bends). To explain this observation, which conflicts with the results in Fig. 4*a*, we note that GCN4 dominates the electrophoretic properties of GCN4–DNA complexes; protein-binding induces remarkably large bandshifts of all DNA fragments examined (from 19 to 350 bp). Since the protein is overwhelmingly rich in acidic residues,

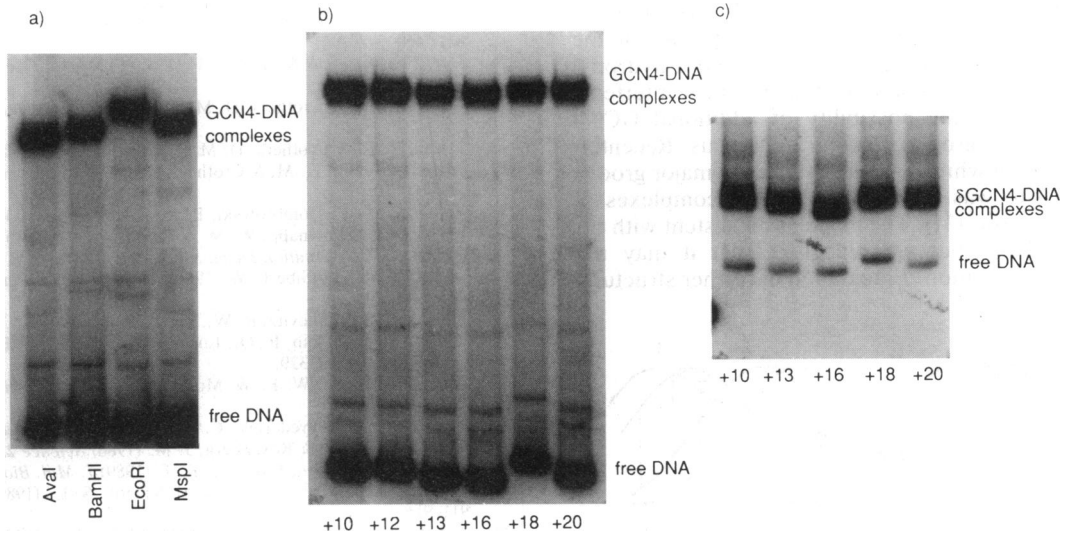


FIG. 4. Gel electrophoretic assays of GCN4-induced DNA bending. (*a*) Circular permutation assay. GCN4-bound circularly permuted DNA fragments were electrophoresed until xylene cyanol had migrated roughly 12 cm. Restriction enzymes with unique cutting sites were used to vary the position of the binding site relative to the molecular ends of a 195-bp fragment; the binding site pseudo-dyad axis is positioned 19 bp from the nearest end for *Ava* I, 36-bp from the same end for *Bam*HI, 78 bp for *Eco*RI, and 159 bp for *Msp* I. (*b*) Relative direction of bending assay. GCN4 binding-site/adenine-tract fusions differing by the length of intervening DNA were bound by GCN4 and electrophoresed until xylene cyanol had migrated roughly 17 cm. The number below each lane (+10, +12, . . .) corresponds to the length of a spacing linker cloned between the two loci. (*c*) Bandshifts with δGCN4, a truncated GCN4 derivative. δGCN4 (described in *Materials and Methods*) was bound to a subset of the fusion constructs and electrophoresed as described until xylene cyanol had migrated roughly 17 cm.

severe band retardation cannot be attributed to the effective charge of the complex at electrophoretic pH of 8.3 (42). Unlike globular proteins, such as *E. coli* catabolite gene activator protein and *lac* repressor, GCN4 may have an extended shape; the apparent molecular mass of the protein by size-exclusion chromatography is approximately 180 kDa (data not shown), considerably larger than the 60-kDa molecular mass of the dimer calculated from the amino acid sequence (43, 44). The structure of DNA-bound GCN4, and not a protein-induced DNA bend, may be responsible for the binding-site position-dependent gel mobility of circularly permuted fragments; effectively, the protein may simulate a branch point on the DNA molecule during electrophoresis.

Support for this argument comes from bandshifts with a truncated GCN4 derivative. A 65-amino acid C-terminal fragment consisting of only the DNA-binding and protein-dimerization activities, termed δGCN4, induces only a small bandshift; protein-bound circularly permuted DNAs yield only minor variations in gel mobility (data not shown), and the mobilities of protein-bound adenine-tract fusion constructs parallel the anomalous mobilities of the corresponding free DNAs (Fig. 4*c*). While these observations do not rule out the possibility that δGCN4 binds differently than intact GCN4, primer extension experiments with partially truncated GCN4 derivatives define a binding domain comparable in size to the full thermodynamic binding domain required by intact protein. In summary, we conclude that GCN4 induces little or no bending in its DNA binding site; the modest electrophoretic anomalies in Fig. 4*b* are ascribed to the dependence of mobility on the position of binding of a relatively nonglobular protein.

Fig. 5 summarizes our data. The thermodynamically defined binding domain spans 18 bp including a 9-bp adenine tract that may contribute to GCN4 binding in part through formation of an alternative DNA conformation. GCN4 appears to contact some portion of every base pair within a 14-bp region. The chemical modification results are consistent with a model for the GCN4–DNA complex in which the DNA-recognition motif of GCN4 lies in and contacts the major groove and backbones of the double helix over nearly one and a half turns. Since the protein binds as a dimer of identical subunits to a DNA site with pseudo-twofold symmetry, it is likely that each subunit contacts half of the binding domain. Because GCN4 does not yield a hydroxyl radical footprint but methylation of adenosines interferes with protein binding, the possibility of additional GCN4 contacts in the minor groove remains ambiguous. Recently, a structural model in which α-helices track the major groove of the binding site was proposed for DNA complexes of leucine-repeat proteins (37). The model is consistent with the data presented here, but we recognize that it may not represent a unique solution. Elucidation of further structural detail of the GCN4–DNA complex must await crystallographic and spectroscopic analyses.

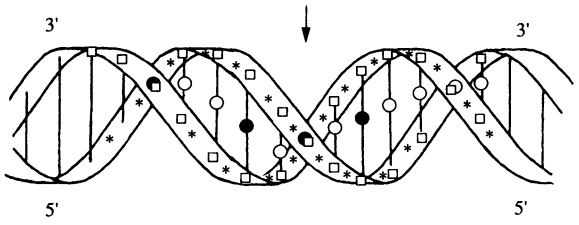


FIG. 5. Summary of the GCN4–DNA interaction data, showing a double helical projection of the chemical modifications that reduce GCN4 binding affinity: *, ethylation of phosphate oxygens; □, eliminated nucleosides; ●, methylation of guanines in the major groove; ○, methylation of adenines in the minor groove. Arrow identifies the central base pair.

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