DNA Binding Specificities of YPF1, a Drosophila Homolog to the DNA Binding Subunit of Human DNA-dependent Protein Kinase, Ku*

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YPF1, a heterodimeric protein from Drosophila melanogaster, is a homolog to Ku, the DNA binding subunit of human DNA-dependent protein kinase. This kinase is crucial in transcriptional activation, V(D)J recombination, double-strand break repair, and both topoisomerase and helicase activities. To investigate functional homology between YPF1 and Ku, we examined DNA binding properties of YPF1. Like Ku, at 100 mM KCl, YPF1 binding has no detectable DNA sequence specificity, requires a DNA terminus, and has a concentration-dependent stoichiometry consistent with subsequent translocation along DNA. YPF1 differs from Ku by having a 105-fold higher affinity. At 400 mM KCl, YPF1 still prefers DNA termini but shows binding specificities not observed previously with Ku. In descending order of affinity, YPF1 binds to: specific DNA sequences with a specific polarity and spacing relative to DNA termini; nonspecific linear DNA; and circular DNA. At this higher ionic strength, binding stoichiometry is concentration independent, indicating that YPF1 remains bound to ends. These results demonstrate a strong functional homology between YPF1 and Ku at physiological ionic strength. The strong binding of YPF1 has also allowed us to detect underlying binding specificities that may be specific to YPF1 and its function.

Ku, a protein originally identified as an autoantigen associated with some forms of human systemic lupus (1), is now known to be an abundant protein in organisms that range from yeast to humans. The human protein, variously referred to as Ku, NF1V, TREF, PSE1, EBP-80, Ki, p70/p80, p70/p86, and many other names (1–10), is the DNA binding subunit of DNA-dependent protein kinase, a kinase composed of both Ku and a large catalytic subunit, p350 (11, 12).

The DNA-dependent kinase phosphorylates many proteins. It was initially implicated in transcriptional regulation because it phosphorylates several transcription factors (Sp1, Oct-1, Oct-2, c-Myc, p53, and the large T-antigen of SV40) as well as the carboxyl-terminal domain of RNA polymerase II (13–18). Ku, and by implication the kinase, have since been shown to activate transcription and to be necessary for repair of radiation-induced double-stranded breaks in DNA, for V(D)J recombination of immunoglobulin genes, and for the activities of topoisomerase II and human helicase II (19–22).

A study of proteins that bind to the yolk protein 1 (Yp1) gene of Drosophila melanogaster led to purification of an abundant Drosophila protein called yolk protein factor 1 (YPF1),1 subsequently shown to be a homolog of Ku (23). Early studies showed that in binding buffers containing 400 mM KCl, YPF1 binds specifically to the HBS1 site located a short distance downstream of the Yp1 promoter (24). When this site is mutated, steady state levels of Yp1 mRNA decrease, suggesting that HBS1 is a regulatory site and that YPF1 may regulate transcription from this site (25).

In this study, we investigate the DNA binding specificity of YPF1. This specificity is crucial to the function of Ku because both kinase activity and assembly of the holoenzyme depend on binding to DNA (26). Two hallmarks of Ku binding are its DNA sequence independence and its requirement for a DNA terminus. This DNA terminus can be either an end where the phosphate backbone of both strands are broken or a transition from double- to single-stranded structure (2, 27–30). This transition, in turn, can be either a nick or gap in one strand or a fork separating the two strands. Another feature of the binding interaction is that once Ku has bound a terminus, it then can translocate along the DNA helix (7). These binding specificities are likely to be related to the unusual DNA structures at which Ku functions, namely those occurring during recombination, transcription, repair, and replication.

We report here that YPF1 shares many DNA binding properties with Ku but also has substantial differences. In the expected physiological range of ionic strength, like Ku, YPF1 binds nonspecifically to DNA. Also like Ku, a DNA terminus is required, and the protein:DNA stoichiometry increases as protein concentration is increased. However, unlike Ku, this binding is extremely tight (Kd ≈ 5 × 10−16 M), approximately 105-fold higher than for Ku (2). Higher ionic strength reveals underlying DNA sequence and structural specificities of the binding interaction. These differences suggest that the two related proteins may have different DNA specificities or function differently in response to cellular cues.

MATERIALS AND METHODS

DNAs—Restriction enzymes (New England Biolabs), calf intestine phosphatase (Boehringer Mannheim), and T4 polynucleotide kinase (New England Biolabs) were used according to standard protocols (31). The terminal phosphates of restriction fragments and oligonucleotides were labeled with T4 kinase and [γ-32P]ATP (6000 Ci/mmol; DuPont NEN). Plasmid pSPYP1 contains Yp1 sequence from −91 to +254 with its terminal restriction sites destroyed by the cloning procedure (23, 32). Therefore, Yp1 restriction fragments extending to −91 or +254 con-
tained a few base pairs from the cloning vector polylinker. These base pairs had no specific YPF1 binding affinity (Fig. 1B). HBS1 and HBS2 fragments containing pBR322 sequences downstream of the specific binding sites were generated by digesting pYP1-5’ (33) with Styl and either NaeI, BanII, or EcoO109I, thereby adding 26, 96, or 148 base pairs of pBR322. HBS1 duplex used in DNA binding experiments was made by annealing two oligonucleotides (5’-AGCTTGACTCCTACGTGTTATCGGGCCGGTTTAAAG-3’ and 3’-ACGTAGAGATACATTAGCCTACGGCGAAATTACG-5’) with sequence unrelated to either HBS1 or HBS2.

Mobility Shift Assay—The purification of YPF1 from Kc0 Drosophila cultured cells and the binding assays were as described previously (24, 25) unless otherwise noted. When binding reactions were complete, an excess of HBS1 DNA was added as a specific competitor, and incubation was continued for an additional 3 min. After electrophoresis in 6% nondenaturing polyacrylamide gels, 32P-labeled DNA was visualized by autoradiography and quantitated with a PhosphorImager (Molecular Dynamics) after dying the gels. To identify DNA in a shifted complex, the complex was eluted from a gel slice overnight at 37 °C in 0.5 mM ammonium acetate and 1 mM EDTA; then DNA was purified, and its size was determined by gel electrophoresis (31).

Binding Competition Assays—DNA binding competition was done with the following changes to the mobility shift assay. Unlabeled competitor DNAs were added to the binding reaction together with radiolabeled HBS1 DNA. Specific competitor was not added after the binding reaction.

Off-rate Determination—After forming YPF1/DNA complexes, an excess of unlabeled HBS1 competitor was added to the binding reaction. Aliquots were taken at various times thereafter and assayed with the gel binding assay. The natural log of the ratio between the concentration of protein-DNA complex and the initial concentration was plotted versus the time elapsed since adding excess HBS1 (24). In this form, the slope is equal to −koff and to = 0.693/koff.

RESULTS

YPF1 Has Dramatically Different DNA Binding Specificities at 100 and 400 mM KCl—Under some conditions, YPF1 binds specifically to the HBS1 DNA site (+150 to +179) in the Yp1 gene (24, 25). To explore this binding specificity, purified protein was incubated with Yp1 restriction fragments at each of two ionic strengths, and then binding was assayed by electrophoretic mobility shifts in nondenaturing gels. At the lower and more physiological ionic strength of 100 mM KCl, YPF1 bound nonspecifically to all DNA fragments, including those with no HBS1 site (Fig. 3A). Increasing YPF1 concentration caused all DNA fragments to shift into complexes of still lower mobility, implying that several YPF1 molecules bind to a single DNA molecule or that YPF1 can link several DNA molecules together (Fig. 3A). We conclude that at 100 mM KCl, YPF1 binds with no detectable DNA sequence specificity and has a binding stoichiometry that depends on YPF1 concentration. These binding characteristics are similar to those of the Ku protein at 100 mM KCl (7) and indicate that like Ku, YPF1 may bind to DNA termini with no sequence specificity and then translocate along the DNA helix.

Confirming earlier indications of sequence-specific binding at the higher ionic strength of 400 mM KCl, YPF1 binds only to a DNA fragment containing HBS1 (Fig. 1B, fragment b+c). In contrast to results at 100 mM KCl, a single binding complex was formed throughout a broad range of YPF1 concentration. At 100 mM KCl, a 5-fold increase in concentration above the level producing the first detectable shifts led to approximately one-half of the DNA occurring in many supershifted complexes (Fig. 3A, lanes 3 and 5). At 400 mM KCl, a 250-fold increase above the level producing the first detectable shifts gave no detectable, supershifted complex (Fig. 1B, lanes 2 and 6). Therefore, in contrast to results at the lower ionic strength, at 400 mM YPF1 binds with sequence specificity and a binding stoichiometry that is insensitive to substantial changes in YPF1 concentration.

These are binding properties not observed previously for the homologous human Ku protein. They indicate that at 400 mM KCl, an underlying DNA binding specificity is revealed when non-specific interactions with DNA are reduced.

At 400 mM KCl, Tight Binding by YPF1 Requires a Specific Sequence Near a Downstream DNA End—Assays of other restriction digest sites showed that YPF1 binds not to one but to at least two sites in the Yp1 gene: HBS1 and HBS2 (Fig. 2; fragments c and b, respectively). For example, binding reactions with one digest produced two shifted bands: one containing only HBS1 and another containing HBS2 linked to upstream DNA (Fig. 2A; upstream relative to the direction of Yp1 transcription). A different restriction digest of the same DNA showed that HBS2 and not the upstream DNA binds YPF1 (Fig. 2B). Although YPF1 binds specifically to both sites, the sequences have no apparent homology (Fig. 3B). In this and all other mobility shift experiments, the identity of DNA in the shifted complex was confirmed using gel electrophoresis to identify the DNA extracted from a shifted complex.

In contrast to most proteins that bind specific DNA sites, a specific double-stranded DNA sequence is insufficient for YPF1 binding at 400 mM KCl. Instead, a nearby DNA end also is required. Adding DNA downstream of HBS1 reduced binding to undetectable levels (Fig. 2, A and B, fragments c versus c+d+e). This end-dependent inhibition also occurs with HBS2 (Fig. 3A). Using YPF1 structure 4 versus structures 6–9). The simplest interpretation is that sequence-specific binding requires a DNA end immediately downstream of the recognition sequence.

The DNA end requirements for binding inhibition were explored in the binding experiments summarized in Fig. 3A. (a) As little as 8 base pairs at the downstream end of HBS1 is sufficient to block detectable binding to HBS1 or to a longer fragment with HBS1 at its downstream end (Fig. 3A, structures 1, 2, 5, and 6; Figs. 1B, 2C, fragments b+c versus b+c+d). (b) Inhibition of binding appears independent of the length of downstream DNA added over a range from 8 to 148 base pairs (Fig. 3A, structures 2, 3, and 7–9).

![Figure 1](image.png)

**Fig. 1.** YPF1 binding specificity at two ionic strengths. At the top is a map of segments of the Yp1 DNA. The arrow indicates the transcription initiation site, the black box indicates HBS2 (+109 to +149), and the gray box, HBS1 (+150 to +179) binding sites. 32P-Labeled restriction fragments (each fragment at 10 pmol) used in the mobility shift experiments are shown below the map. A, autoradiogram of a mobility shift assay of binding at 100 mM KCl. Lanes 1–6 contain 0, 9, 30, 90, 150, 210, and 300 pmol of purified YPF1, respectively. B, an autoradiogram of a mobility shift assay of binding at 400 mM KCl. Lanes 1–6 contain 0, 60, 180, 450, 1500, and 15,000 pmol of YPF1, respectively. DNA fragments in the bands are indicated by lowercase letters, with parentheses indicating shifted bands.
inhibition does not depend on the sequence of the downstream DNA. Bacterial plasmid DNAs were as effective as Yp1 DNA (Fig. 3A, structures 7–9). (d) Binding inhibition does not appear to depend on the structure of the downstream end. Blunt, 3′-overhang, or 5′-overhang ends were equally effective (Fig. 3A, structures 7–9, respectively).

These data also indicate that YPF1 binds to its recognition sequence with a specific polarity. Whereas binding was inhibited by DNA sequences downstream of HBS1, a very long DNA sequence added upstream had no effect (Fig. 3A, structures 5 and 10). Therefore, at 400 mM KCl, YPF1 binds with a specific polarity to a specific DNA sequence, and a nearby downstream DNA end is crucial for strong binding.

Relative Binding Affinities for Specific and Nonspecific Linear DNAs and for Circular DNAs at 400 mM KCl—We used DNA competition experiments to measure relative binding affinities at 400 mM KCl. Purified YPF1 was incubated with mixtures of 32P-labeled HBS1 and unlabeled competitor DNAs for 2 h, a period 8-fold longer than necessary to reach binding equilibrium with HBS1 (data not shown). Two mobility shift gels shown in Fig. 4A illustrate the approximately 100-fold different competitive effect of two DNAs, one a linearized bacterial plasmid with HBS1 at one end and the other a similar linearized plasmid without HBS1. The quantitative effects of several different DNA sequences and structures were examined. Competition curves demonstrated that moving HBS1 from the downstream end to an internal position in a linear DNA reduces affinity by 100-fold (Fig. 4B). This is similar to the effect of removing the HBS1 end from linear DNA (Fig. 4A) and reduces DNA affinity for YPF1 to the level of nonspecific linear DNAs, for example, linearized bacterial plasmid pBR322 or total Drosophila chromosomal DNA digested with HindIII restriction enzyme. Circular DNA has a 30-fold lower affinity than nonspecific linear DNA or approximately 3000-fold below linear DNA with HBS1 at the downstream end (Fig. 4B). We conclude that there are at least three classes of affinities at 400 mM KCl. In descending order of affinity, they are: specific binding sites located a few base pairs upstream from a DNA end; nonspecific linear DNAs or specific binding sites more than a few base pairs upstream of a DNA end; and circular DNAs.
Association and Dissociation Rates Make Approximately Equal Contributions to the Difference between Specific and Nonspecific Binding at 400 mM KCl—The stabilities of complexes between YPF1 and either specific or nonspecific linear DNAs were investigated at 400 mM KCl (Table I). The dissociation rates were examined by binding YPF1 to radiolabeled DNA fragments, adding unlabeled HBS1 in vast excess, and then using mobility shift assays to measure the time-dependent decrease in radiolabeled complex (24). $k_{\text{off}}$ increases when additional DNA is included downstream of HBS1 and becomes approximately the same as for nonspecific DNA (Table I). Although the direction of the change is as predicted by the competition experiments described above, the differences are approximately 6-fold rather than 100-fold. This indicates that the affinity difference for specific and nonspecific DNA at high salt concentration is due to approximately equal contributions from association and dissociation rates.

Association and Dissociation Rates Show That Sequence-specific Binding Does Not Occur at 100 mM KCl and That Association Rates Are Proportional to DNA End Concentration—YPF1 forms an extremely stable complex with HBS1 at 100 mM KCl ($K_D \approx 5 \times 10^{-10}$ M) and a complex 8 orders of magnitude less stable with the synthetic copolymer, poly(dA)-poly(dT) (24). Based on this observation, it was surprising that the qualitative mobility shift experiments shown in Fig. 1A indicate that different restriction endonuclease fragments of naturally occurring DNA have similar or nearly identical affinities for YPF1. The unusual helical structure of poly(dA)-poly(dT) (34) may account for its drastically lower affinity, yet smaller differences between the fragments of naturally occurring DNA may not have been detected in the qualitative assay. To investigate this possibility, we used quantitative assays of the dissociation rates and apparent association rates.

No sequence specificity was detected in binding to linear DNA at 100 mM KCl. The dissociation rates are essentially the same for DNA without the HBS1 site and with that site either at the end or in the middle of DNA fragments (Table I). This rate is approximately 4 orders of magnitude lower than for interactions with the specific site at the higher ionic strength (from $2.3 \times 10^{-7}$ s$^{-1}$ to $1.4 \times 10^{-3}$ s$^{-1}$). DNA competition was used to examine the relative association rates (Fig. 5). $^{32}$P-Labeled HBS1 was mixed with different concentrations of unlabeled DNA. Pure YPF1 was then added, and reactions were allowed to proceed for 16 h. Because the dissociation rates are very low and essentially the same for all linear DNAs examined, this experiment is likely to measure the relative rates of association. Whether or not they contain HBS1, all of the linear DNAs tested compete equally well on a molar basis for YPF1 binding (Fig. 5). We conclude that the association and dissociation rates for all linear DNA species are approximately the same and, therefore, that the equilibria are approximately the same ($K_D \approx 5 \times 10^{-10}$) at this low ionic strength.

Although there is no apparent sequence specificity to YPF1 binding at 100 mM KCl, association appears to be in proportion to the concentration of DNA ends. As an indication of this, the competition by predominantly circular pBR322 plasmid is 9-fold lower than the predominantly linearized form of the same plasmid (Fig. 5). More directly, digestion of pBR322 with HinfI, which cuts at 10 sites, results in 10-fold better competition than linearized, full-length pBR322 (Fig. 5).

We conclude that the extremely stable binding between YPF1 and DNA at 100 mM KCl has no detectable specificity for particular DNA sequences but does have an association rate that is proportional to the number of DNA ends rather than the total mass of DNA. This association rate dependence on DNA ends together with the protein concentration-dependent stoichiometry of the interaction is consistent with the DNA-binding model for the Ku protein, namely, binding initially to a DNA end and subsequently translocating to an internal position in the DNA.

**DISCUSSION**

Previous studies have shown that the Drosophila YPF1 and the human Ku proteins share many properties. Both are abundant DNA binding proteins that have similar heterodimeric subunit structures and molecular weights (24, 35). The structural similarity extends to the sequence level because the sequences of the small subunits are 24% identical and 51% similar over their entire 631 amino acid length, and those of the large subunits yield the same antigenic epitopes (23). Furthermore, the roles of the subunits in DNA binding are similar between the two proteins. The small subunit of each can bind DNA independently of the large subunit, and both small and large subunits are in contact with DNA when the heterodimers are bound to DNA (23).

In this report, we have tested the functional similarity between YPF1 and Ku by examining the DNA binding specificities of YPF1. Previous studies with Ku have shown that binding DNA is necessary for assembly of Ku into the DNA-dependent protein kinase holoenzyme. DNA binding by Ku is also necessary for activity of the kinase. Thus, DNA binding is tightly linked to Ku function, and examining binding properties of YPF1 should test similarity in crucial functions.

**Nonspecific Binding to DNA at 100 mM KCl**—We have used
the mobility shift assay to examine YPF1 binding under the same ionic strength conditions as used previously for Ku. The Ku studies led to a model that it binds initially to double-stranded DNA termini with no DNA sequence specificity, and then translocates along the DNA helix (1, 2, 4, 7, 12, 29, 30). We find that at this ionic strength, YPF1, properties match this model for Ku binding and translocation. However, we also find that YPF1 dramatically differs from Ku in its binding affinity. YPF1 affinity is 105-fold higher.

As with Ku, YPF1 binds with no DNA sequence specificity among the Drosophila and Escherichia coli DNAs tested either in qualitative assays (Fig. 1) or in quantitative assays of association and dissociation rates (Table I). Previously, it was observed that YPF1 bound much less strongly to poly(dA)-poly(dT) than to naturally occurring DNA (24). However, the current study indicates that poly(dA)-poly(dT) is an exceptional case, perhaps due to its unusual secondary structure (34).

Like Ku, YPF1 prefers DNA termini. Quantitative data indicate that binding to linear DNA is at least 9-fold stronger than to circular DNA (Table I). However, this is a lower limit for selectivity because the circular DNA preparation used contained a small amount of linear DNA as well as larger amounts of nicked and gapped circles that would bind Ku as tightly as would double-stranded termini (4, 27). A better measure of end preference is the observation that binding is proportional to the number of DNA termini (Fig. 5), an observation also made with Ku (4, 7, 28).

Finally, both YPF1 and Ku form multimeric complexes with DNA under conditions of protein excess (Fig. 1; Refs. 7, 11, 12, 29, and 30). This observation, along with an inherent recognition of DNA termini, is consistent with the translocation of Ku and YPF1 away from the DNA terminus.

The only difference we found between Ku and YPF1 at 100 mM KCl is its extremely high affinity for DNA ends. This affinity is approximately 105-fold higher than the sequence-specific binding affinity of regulatory proteins such as LacI, CRP, and AraC of E. coli and the sequence-nonspecific binding of Ku protein itself (27, 36–38).

Taken together, the similarities between in vitro binding activities of YPF1 and Ku give further support to the hypothesis that the two proteins are homologous, not only in structure but also in function. Since binding of Ku to DNA is critical for the activity of DNA-dependent protein kinase, the similarity of YPF1 binding specificities suggests that it also associates with a Drosophila protein kinase and serves to locate it at appropriate substrates. Interestingly, a DNA-dependent protein kinase has recently been detected in Drosophila embryos (39), a stage at which YPF1 and its transcript have been shown to be most highly abundant (23, 25) and a stage at which replication activity is very high.

Specific Binding to DNA at 400 mM KCl—The extremely tight binding at 100 mM suggested that YPF1 has interactions with DNA beyond those of Ku and may recognize additional structures in DNA. To examine this possibility, we studied its DNA binding at a higher ionic strength that is likely to reduce nonspecific interactions between YPF1 and DNA.

At 400 mM KCl, YPF1 has binding specificities not previously observed with other members of the Ku family. (a) YPF1 binds to the ends of long linear DNA molecules with an apparent stoichiometry of 1:1, independent of large changes in YPF1 concentration. (b) YPF1 binds most tightly to a particular structure at a DNA end: a specific sequence with a specific polarity to a DNA end and at a specific distance from that end. This suggests that at the higher ionic strength, YPF1 makes contact with a specific DNA site and also with the adjacent downstream terminus. Such a YPF1 structure must be fairly rigid because addition of as few as 8 base pairs to the end of the DNA abolishes binding. However, at lower ionic strength, this rigidity must be lost because YPF1 recognizes DNA ends and appears to translocate to internal positions, thus allowing more than two protein complexes to interact with a single linear DNA molecule. It is worth noting that even the weakest binding specificities we have observed are in the range of proteins that operate at specific DNA sites. Thus, it is likely that even these interactions are physiologically relevant to YPF1 interaction with DNA.

These observations indicate that more subtle interactions with DNA may take place, although the general model for Ku binding still applies to YPF1. The in vivo structures of the DNA ligands for YPF1 and Ku are unknown. In the case of Ku, there is indirect evidence that it is a non-helical intermediate of reactions that require both single and double strands. These reactions include replication (10, 21), transcription (3, 40–42), DNA repair of double-stranded breaks (39, 43, 44) and V(D)J recombination (20, 45, 46). The diverse nature of these reactions is most likely the result of the association of Ku with many different regulatory proteins, such as the DNA-dependent protein kinase p350 (11, 12), phosphatase PPA2 (47), TBP (40), and TRAC (48), as well as the helicase and somatostatin receptor activities it has on its own (21, 47).

Additional insight into the potential for modifying the DNA ligand specificity of YPF1 is provided by the isolation and characterization of IRBP (49, 50), recently identified as the small subunit (p70 or β subunit) of YPF1 (50). In contrast to the binding behavior of the heterodimeric YPF1 protein that we report in this study, the purified p70 subunit binds to specific DNA sequences at low salt concentration and with no apparent requirement for an adjacent downstream terminus. The specific DNA sequences bound are the terminal inverted repeats of the P-element transposon, target sites consistent with some of the proposed functions of Ku. It is most likely that the different binding specificities observed are due to the modulating effects of the p86 subunit present in the heterodimeric YPF1, although they could be due to subtle effects of binding conditions and DNA sequence.

As mentioned above, Ku appears to play a crucial role in such diverse cellular processes as protein phosphorylation, immunoglobulin gene maturation, DNA repair, site-specific recombination, DNA unwinding, and signal transduction. As a means of identifying the functions of the Drosophila Ku, the p70 subunit has been cytologically mapped (23, 50). Recent mutant rescue results demonstrate that the cytological location is in the mus309 gene at position 86E2–3 rather than the 34C position we reported (23, 51). This localization allowed Drosophila molecular genetics to be used to determine that double-strand DNA break repair is one of the in vivo functions of the smaller subunit and, presumably, of the heterodimeric YPF1/Drosophila Ku (51).

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