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# Protein/DNA crosslinking of a TFIID complex reveals novel interactions downstream of the transcription start

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## ABSTRACT

**A protein–DNA complex containing TFIID has been analyzed by crosslinking. The TBP subunit of TFIID crosslinked to the TATA element but not to any of the regions further downstream which were tested. A 150 kd polypeptide, which corresponds in size to one of the TBP-associated factors (TAFs), crosslinked to a region between +10 and +15 and a second region between +35 and +47. Another polypeptide of greater than 205 kd (also a potential TAF) crosslinked preferentially to the region between +35 and +42. The +10 to +15 region has been recently implicated in hsp70 promoter recognition by TFIID, and the most downstream contacts overlap with the region where RNA polymerase II pauses on the hsp70 promoter in noninduced cells. Crosslinking revealed that as the salt concentration was increased, the TBP interaction was largely unaffected whereas the protein/DNA interactions downstream of the TATA element were disrupted. We propose that during the formation of a transcription complex, TATA-dependent interactions could be disrupted in the vicinity of the start site and the region immediately downstream. A protein contact downstream of +35 might function in pausing polymerase.**

## INTRODUCTION

The hsp70 gene of *Drosophila* has been an excellent model system for studying the mechanism of transcriptional regulation. Work on this gene has highlighted the importance of protein–DNA interactions in the region downstream of the transcription start site. The structure of the promoter in normally growing cells is established so that the promoter can be rapidly induced in response to stress. A transcriptionally engaged RNA polymerase II has paused in a region approximately 20 to 40 nucleotides downstream from the start site (1, 2, 3, 4, 5, 6). Deletion analysis has revealed that breakpoints in the region downstream of the transcription start reduce both the levels of paused polymerase that are present prior to heat shock and the levels of induction after heat shock (4). Biochemical analyses have shown that the binding of transcription factor IID to the hsp70 promoter is strongly influenced by sequences at the transcription start and in the region extending approximately 30 base pairs farther downstream (7, 8). The RNA polymerase II and TFIID interactions of the type described for hsp70 are not restricted to this gene. Paused polymerase has been detected on both heat shocked and nonheat-shocked genes in both *Drosophila* and mammalian cells (2, 3, 9) and TFIID contacts at the transcription start have been found to be important in binding for the histone H3 and H4 genes (10, 8).

We are interested in understanding how the interactions of different proteins in the region downstream of the transcription start site are integrated with each other. Our footprinting analysis of immunopurified TFIID showed that the protein contacts the region from −40 to +35 in the hsp70 promoter (7). This overlaps with the region where RNA polymerase II is found to pause and with the region where the initiation complex assembles on the promoter. The manner in which these two proteins and others interact in the region downstream of the TATA element are likely to be important in the transcription mechanism.

To study the interactions of multiple proteins in the promoter region of the hsp70 promoter, we have used a crosslinking technique that was previously developed for the analysis of a TATA-dependent protein–DNA complex (11). We now know that this TATA-dependent protein–DNA complex contains transcription factor IID (7). Here, we use the crosslinking technique to analyze the protein–DNA interactions associated with this TFIID-containing complex.

## MATERIALS AND METHODS

### DNA's/plasmids used

The hsp70 promoter subclones contain a version of the promoter originally from the 87C locus. The subclones to used to produce the labeled fragments for binding assays contain sequences spanning the region from −190 to +89 (12, 11). The TATA deletion was constructed by removing the sequence between the HaeII restriction sites flanking the TATA box (12, 11).

### Preparation of partially purified TFIID

TFIID was partially purified as described by Purnell and Gilmour (8). The fraction used corresponds to the fraction eluted from phosphocellulose with 0.5M KCl buffer.

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### DNase I footprinting

Protein preparations were allowed to bind end-labeled promoter fragments for 20 min in a 25 μl volume of 20 mM HEPES (pH 7.6), 5 mM $MgCl_2$, 40 μM EDTA, 4% glycerol, 0.9 mM DTT, 20 ng/μl HaeIII-cut *E.coli* DNA, 88 mM KCl at 25 °C. At this time, 2.5 μl of either 88 mM or 2.97 M KCl in 20 mM HEPES (pH 7.6), 5 mM $MgCl_2$, 40 μM EDTA, 4% glycerol was added and the incubation was continued for another ten minutes. Digests were performed by adding DNase I in 50 mM NaCl, 20 mM Tris·HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM $CaCl_2$, 100 ng/μl acetylated BSA. The high salt samples were digested with 2 μl DNase I at 0.5 U/μl. The low salt, no protein control was digested with 1 μl DNase I at 0.01 U/μl. The low salt, protein-containing sample was digested with 2 μl DNase I at 0.01 U/μl. After allowing the digests to proceed for 30 sec at 25 °C, the reactions were stopped by the addition of 50 μl 0.5% sarkosyl, 5 mM EDTA, 40 ng/μl sonicated salmon sperm DNA and then purified by phenol extraction and ethanol precipitation.

### Purification of RNA polymerase II

RNA polymerase II was isolated from the 600 mM KCl fraction off the DE52 column used in the purification of TFIID (8). The remainder of the purification was adapted from a previously published procedure (13). The activity of RNA polymerase II was assayed by another previously published procedure (14).

The DE52 fraction was diluted with Buffer G (25 mM HEPES (pH 7.6), 0.1 mM EDTA, 15% glycerol, 1 mM DTT, 0.5 mM PMSF) to a conductivity of 200 mM KCl/Buffer G. This was then loaded onto a heparin−sepharose CL-6B column pre-equilibrated in 200 mM KCl/Buffer G and washed with the same solution. The RNA polymerase II was then eluted with a step to 500 mM KCl/Buffer G.

The protein peak was pooled, diluted with Buffer G to a conductivity less than or equal to 300 mM KCl/Buffer G, and loaded at 1 ml/min onto a MonoQ HR5/5 FPLC column equilibrated in the same solution. After a wash, the RNA polymerase II was eluted with an 18 ml gradient from 300 mM to 600 mM KCl/ Buffer G.

Using this method, RNA polymerase II activity eluted at approximately 500 mM KCl/Buffer G, corresponding to the tail end of a tripartite 280 nm absorbance peak. It was judged to be approximately 80% pure by SDS-PAGE analysis.

### DNA preparation for crosslinking

Radiolabeled DNA's for use in crosslinking assays were prepared by pulse-chase primer elongation on a single-stranded M13 phage DNA template (11). Oligonucleotide primers (see Figure 1) were annealed to the single-stranded, circular phage DNA for several hours at 37°C at a 3:1 molar ratio in 10 mM Tris−HCl (pH 7.6), 1 mM EDTA, 60 mM NaCl. Typically, 3 pmol primer was annealed to 1 pmol M13 template in 5 μl TE, 60 mM NaCl overnight. Limited extension with ($\alpha^{32}$P) TTP was accomplished with the Klenow fragment in the absence of at least one of the nucleotides. The extensions were carried out by adding 1.5 μl 0.1 M Tris−HCl (pH 8), 0.1 M $MgCl_2$; 1.5 μl 100 mM DTT; 3.2 μl $H_2O$; 3 μl ($\alpha^{32}$P) TTP (3 kCi/mmol, 10 mCi/ml); 0.15 μl of each appropriate cold dNTP at 10 mM or TE, and 0.5 μl Klenow fragment (~2.5 U). Incubations were carried out for 15 min at 37°C. Judicious choices for primers and nucleotides for the 'pulse' phase of the extension allowed for a great variety of labeled DNA's. After the pulse, all four nucleotide triphosphates were added in excess both to quench the radioactive TTP and to allow unlimited extension. Half a microliter of unlabeled dNTPs was added from a 10 mM stock along with another 0.2 μl Klenow fragment (~0.4 U) for the cold chase which proceeded for 10 min at 37°C. The resulting double-stranded hsp70 promoters were excised as PvuII/NruI restriction fragments and isolated by polyacrylamide gel electrophoresis.

### UV crosslinking

Proteins contacting DNA were detected using UV crosslinking. The UV crosslinking was performed as previously described (11) except that the reaction volumes and all subsequent steps were doubled in amount.

### Immunoprecipitation of TBP

Tagging of TBP with radiolabeled DNA was first demonstrated by incubating 100 ng of recombinant TBP in 25 μl of 60 mM KCl, 20 mM HEPES (pH 7.6), 5 mM $MgCl_2$, 4 ng/μl HaeIII-cut *E.coli* DNA, 20,000 cpm/μl labeled DNA, 10 mM HEPES(pH 7.6), 0.04 mM EDTA, 0.2 mM DTT, 4% glycerol, 0.04% NP-40. After a 45 min incubation at 25°C, samples were UV irradiated and processed for analysis on SDS-PAGE as previously described (11).

Tests of the antibody for immunoprecipitation were performed by scaling up the binding reaction described above by 3-fold and reducing the radioactive probe to 8000 cpm/μl. After binding and crosslinking, the 75 μl binding reaction was processed with detergent-containing solutions and nucleases as previously described (11). One hundred microliter portions of the final mixture were transferred to fresh tubes and 10 μl of 200 mM EDTA and 1.5 μl of either anti-TBP antiserum or preimmune serum were added. The mixture was incubated on ice for 3 hrs. Fifteen microliters of Pansorbin cells (Calbiochem) were added and the samples were incubated with gentle agitation for an additional hour at 4°C to bind the antibody. The Pansorbin cells were collected by brief centrifugation and then washed 5 times with 1 ml portions of 100 mM Tris−HCl (pH 9.0), 1% sodium deoxycholate, 1 % NP-40, 0.5 M LiCl. The supernatant from the immunoprecipitation was TCA precipitated. The Pansorbin was suspended by triteration during each wash. Both the immunoprecipitation supernatant and immunoprecipitate were analyzed by SDS-PAGE.

Immunoprecipitation of tagged TBP from the TFIID complex was accomplished as follows. Two binding reactions containing 7.4 μg of P-11 fraction (4 μl) and 200,000 cpm of TATA-labeled DNA were set up in 50 μl of 80 mM KCl, 44 ng/μl HaeIII-cut *E.coli* DNA, 30 mM HEPES (pH 7.6), 5 mM $MgCl_2$, 8% glycerol, 0.08 mM EDTA, 1 mM DTT. After 45 min incubation at 25°C and 2 min. of UV irradiation, each sample was processed with the detergent mixtures and nucleases as previously described (11). One microliter of antiserum was added to one sample and 1 μl of preimmune serum was added to the other. Each sample was incubated for 15 min at room temperature followed by 15 min on ice. Two microliters of a 20% suspension of protein G sepharose (Sigma) was added to each sample and the samples were gently rocked for 1 hr at 4°C. The protein G sepharose was collected by brief centrifugation and then washed 5 times with 100 mM Tris−HCl (pH 9.0), 1% sodium deoxycholate, 1 % NP-40, 0.5 M LiCl. The washed pellets were analyzed on SDS-PAGE.

## RESULTS

### Comprehensive survey of the contacts made by the TATA complex on the hsp70 core region

We have recently shown that the protein/DNA complex that forms on the hsp70 promoter when the DNA is incubated with a protein fraction isolated from *Drosophila* nuclear extracts is TFIID. This conclusion is based on the finding that the partially purified complex produces a DNase I footprint that matches the footprint produced by the immunopurified TFIID (7). Moreover, binding assays with hydroxyl radical modified DNA or with mutant DNA's revealed that both the partially purified factor and the purified TFIID make sequence-specific interactions in the TATA element, the start site, +18 and +28 regions (7, 10, 8).

To identify the polypeptides contacting specific regions of the hsp70 promoter, we constructed 6 different versions of the hsp70 core promoter region, each with radioactive thymidine deoxynucleotides incorporated into different positions along the DNA (Figure 1). Five of these were prepared by carrying out controlled DNA polymerization reactions with appropriate synthetic oligonucleotide primers. The sixth primer was prepared as an isolated single-stranded restriction fragment as described previously (11).

Figure 2 presents the results of crosslinking TFIID to the 6 preparations of the hsp70 promoter. In each case, the radiolabeled DNA was incubated with protein and then UV irradiated to generate protein/DNA adducts. Then each sample was treated with nucleases to degrade the DNA and subjected to SDS-PAGE. Those proteins that were tagged with residual radioactive oligonucleotides were detected by autoradiography. Many polypeptides were detected with several appearing regardless of where the radioactivity was positioned in the DNA fragment.

In order to identify TATA-dependent interactions, three binding reactions were compared. The first reaction contained no specific competitor (although it did contain 500 ng of HaeIII-cut *E.coli* DNA). The second reaction contained a specific competitor DNA that spanned the region from −190 to +89 of the hsp70 promoter (wt). This fragment should have inhibited the tagging of any polypeptides that contacted the fragment in a sequence-specific manner. The third competitor revealed which of the contacts on the DNA were TATA-dependent. This fragment spanned the region from −190 to +89, but was deleted of the TATA element (ΔTA).

Several TATA-dependent interactions were detected by the crosslinking analysis. A polypeptide of 42 kd crosslinked to the TATA element. Of the collection of fragments tested, labeling was only observed when the TATA element contained the radioactivity. An additional polypeptide of 26 kd was also lightly labeled and was more apparent in experiments described later (See Fig. 5A). These results are comparable to results that we observed previously for crosslinking with the fragment labeled in the TATA region (11).

A 150 kd polypeptide was found to crosslink specifically to two regions downstream of the transcription start. Contact appeared in the +35 to +47 region and again in the region between +10 and +15. Tagging of this polypeptide was significantly diminished when the radioactivity was localized between +18 and +25. We had hoped to determine if this or any other polypeptide specifically interacted with the start site, so we extended the radioactive region from +15 to −3. The 150 kd polypeptide was still evident, but no additional proteins that interact in TATA-dependent manner were apparent. In fact, enlarging the radioactive region increased the number of polypeptides that were crosslinked nonspecifically to the promoter.

Another polypeptide of greater than 205 kd in size appears to interact specifically with the region between +35 and +42 and perhaps also with the region between +10 and +15. This large polypeptide is more clearly evident in Fig. 5C, and we estimate that it has a size of approximately 250 kd.

### The 42 kd polypeptide can be immunoprecipitated with anti-TBP antiserum

The 42 kd polypeptide is similar in size to TBP so antiserum raised against recombinant TBP was obtained to determine the relationship of these two proteins. We first tested if recombinant TBP could be immunoprecipitated after it had been UV irradiated and tagged by our procedure. Figure 3A shows that recombinant TBP was tagged regardless of whether the radioactivity was incorporated into the TATA element or the downstream region. This lack of specificity reflects the affinity of TBP for random DNA sequences (15, 16). Figure 3B shows that the antiserum,


**Figure 1.** The hsp70 minimal promoter sequence and oligonucleotide primers used for site-specific labeling. The upper two sequences represent the transcribed and nontranscribed strands of the hsp70 promoter. All primers were chemically synthesized with the exception of that designated hsp70 HaeII/HindIII primer. This last primer is derived from a plasmid as previously described (11). Extension of the primers involves addition of nucleotides in the leftward direction of the diagram. The fragments used for crosslinking were derived by cutting the labeled products with NruI and PvuII.

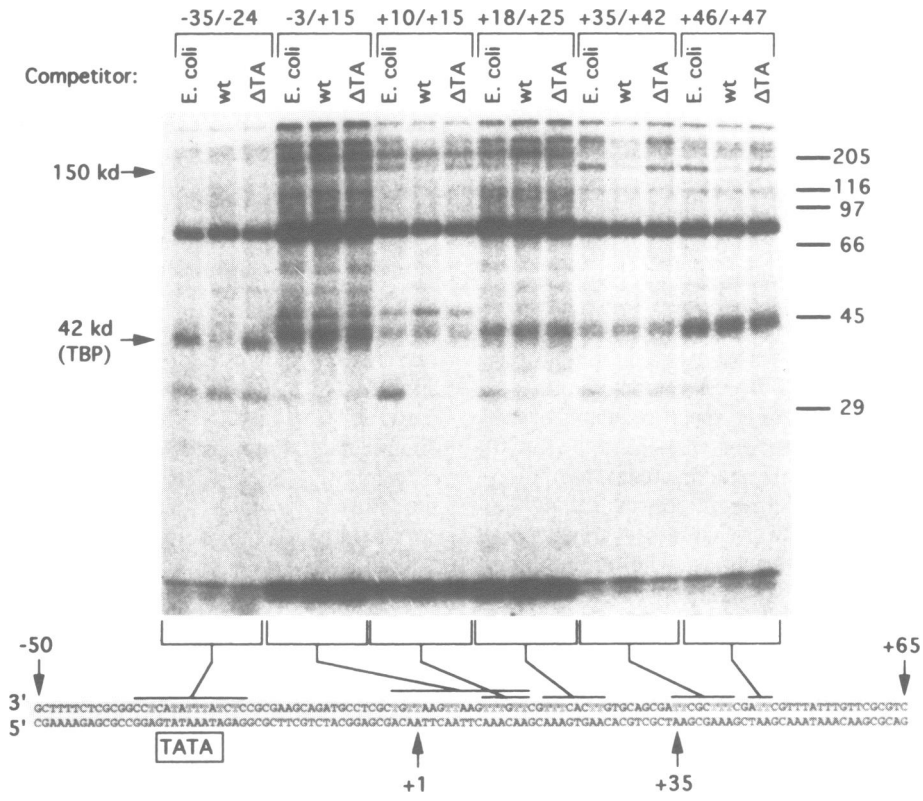


**Figure 2.** Crosslinking survey of TATA-dependent proteins along the hsp70 minimal promoter. For each triplet the overlined sequence is that generated during the first polymerization step and the highlighted T's are those that included the $^{32}P$. The TATA-dependent proteins are competed away by the wild-type competitor (wt) but not by the TATA-deleted competitor (ΔTA). These competitors span the hsp70 promoter from −190 to +89.

but not the preimmune serum, immunoprecipitated the radioactively-tagged recombinant TBP.

The immunoprecipitation was then performed on the polypeptides tagged in the TATA complex by crosslinking to radioactive nucleotides located in the TATA element. The 42 kd polypeptide was immunoprecipitated by the antiserum but not by the preimmune serum indicating that it is TBP (Figure 3C). Additional polypeptides were recovered by both sera and are presumed to be nonspecific.

### The 150 kd polypeptide is not a subunit of RNA polymerase II

When we first reported the crosslinking of a 150 kd polypeptide (11), we suggested that this protein might be the second largest subunit of RNA polymerase II because of the similarity in molecular weights. We have now tested this hypothesis by comparing the mobility of the 150 kd polypeptide to the subunit of purified RNA polymerase II. The 140 kd subunit of RNA polymerase II can be tagged by UV irradiating a mixture of purified RNA polymerase II and radiolabeled DNA. As shown in Figure 4A, the second largest subunit of RNA polymerase II was labeled far more effectively when the RNA polymerase II was incubated with single-stranded DNA than with double-stranded DNA. When the tagged RNA polymerase II was run beside the 150 kd polypeptide that was tagged in the TFIID complex, the mobilities were clearly distinct (Figure 4B). Additional evidence (data not shown) supports the contention that the 150 kd polypeptide in the TFIID complex is not derived from RNA polymerase II. Attempts to immunoprecipitate the 150 kd polypeptide from the TFIID-containing fraction with antibody against RNA polymerase II have failed. Also, no RNA polymerase II activity has been detected in this fraction with a promoter-independent transcription assay.

### Interactions of protein downstream of the TATA element are disrupted by high salt whereas the TBP interaction at the TATA element is not

The finding that the 150 kd polypeptide and the polypeptide greater than 205 kd contacted downstream of +35 was unexpected since DNase I footprinting assays and Exonuclease III protection assays had not detected contacts beyond this point on the nontranscribed strand of the normal hsp70 promoter (11). In addition, neither interference assays nor binding assays with mutant promoters have provided any conclusive evidence that contacts downstream of +35 are involved in binding TFIID (7, 8).

The polypeptide contacts that are only detected by crosslinking may be weaker than the polypeptide contacts that are detected by nuclease protection assays with DNase I or Exonuclease III. To explore this possibility, we tested whether crosslinking of the components of TFIID were affected differently by increasing the ionic strength of the solution. For this analysis, the TFIID complex was first assembled on the DNA and then the ionic strength was increased by adding concentrated potassium chloride. Ten minutes after adding salt, most of each sample was

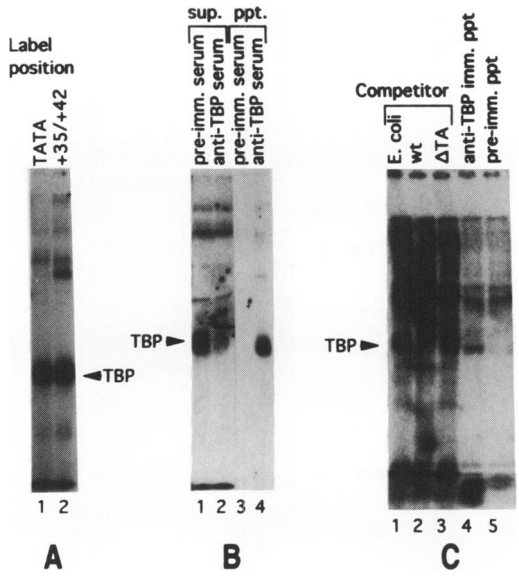


**Figure 3.** Immunoprecipitation of the 42 kd polypeptide tagged at the TATA box verifies its identity as the TBP. (**A**) Tagging recombinant TBP by crosslinking. Recombinant TBP was incubated with two labeled preparations of the hsp70 promoter and then subjected to crosslinking. (**B**) Radiotagged recombinant TBP is specifically precipitated by the anti-TBP immune serum. Immunoprecipitations were performed with preimmune serum (lanes 1 and 3) and anti-TBP antiserum (lanes 2 and 4). TBP is only recovered in the precipitate of the anti-TBP antiserum (lane 4) and depleted from the corresponding supernatant (lane 2). (**C**) The 42 kd polypeptide tagged at the TATA box (lane 3) is precipitated by the anti-TBP serum (lane 4), but not by the pre-immune serum (lane 5). Labeling of the TBP in the TATA complex was accomplished with the hsp70 promoter that was selectively labeled in the TATA element. Lanes 1, 2, and 3 show the polypeptide tagged in the presence of various competitors.

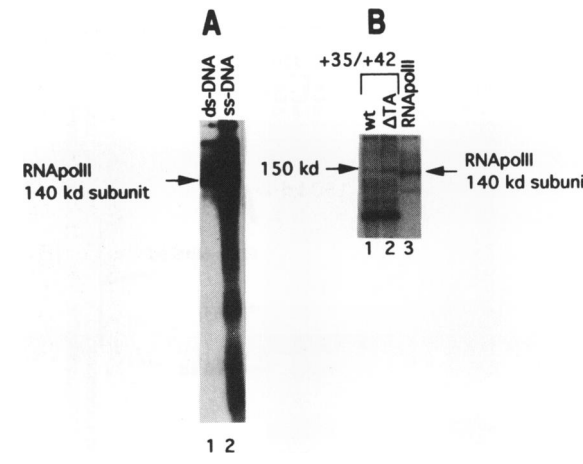


**Figure 4.** The 150 kd peptide tagged downstream of the start site is not the second largest subunit of RNA polymerase II. (**A**) The 140 kd subunit of RNA polymerase II is more efficiently tagged when bound and crosslinked to single-stranded DNA (lane 2) than with double-stranded DNA (lane 1). (**B**) The 150 kd TATA-dependent polypeptide tagged between +35 and +42 (lane 2) migrates more slowly during SDS-PAGE than does the 140 kd subunit of polymerase (lane 3).

UV irradiated and the tagged proteins were analyzed. A small portion was also analyzed by PAGE mobility shift (see below). Figure 5A shows that the interaction of the TBP subunit in TFIID was resistant to increasing ionic strength whereas the interactions of other polypeptides were sensitive. There was no significant reduction in the level of tagged TBP with the addition of KCl up to a final concentration of 350 mM. However, the tagging of the 26 kd polypeptide was significantly reduced by raising the salt to 150 mM. Tagging of the 150 and 250 kd polypeptides contacting the +35 to +42 region decreased above 150 mM KCl (Figure 5C). The 150 kd polypeptide contacting the +10 to +15 region was no longer tagged once the KCl concentration was above 200 mM (Figure 5B).

The insensitivity of the DNA contacts of the TBP subunit to the elevated salt concentrations raised the intriguing possibility that an intact TFIID molecule remained associated with the promoter even though the contacts downstream of the TATA element had been broken. The gel shift assay was used to test the integrity of the TFIID/DNA complex after elevating the ionic strength. No significant change in the mobility of the TFIID/DNA complex was observed as a result of increasing the salt concentration (Figure 6). The loss of intimate contact between these polypeptides and the DNA does not affect the mobility of the protein/DNA complex, implying that they are still associated with the complex as a whole. One would have expected that the dissociation of the 150 and 250 kd polypeptides from the protein/DNA complex would have significantly altered the mobility since the binding of anti-TBP antibody to the TFIID/DNA complex caused a notable change in the mobility of the complex (7, 8). It seems then that at least the largest subunits of TFIID remain associated with the promoter through the TBP/DNA contact even after other contacts have been disrupted.

We were interested in determining if the structural changes that occurred when the TFIID/DNA complex was subjected to high salt resulted in a change in the accessibility of the DNA. The accessibility of the DNA at elevated salt concentrations was measured with DNase I. The complex was assembled in 88 mM KCl and then treated with either 88 or 350 mM KCl for 10 minutes. Figure 7 shows that significant structural changes in the protein/DNA interaction accompanied the high-salt treatment. In contrast to the pattern observed in low salt (lanes 3 and 7), the pattern of DNase I cutting downstream of the TATA element is similar to naked DNA (lanes 4 and 8). This indicates that the accessibility of the DNA downstream of the TATA increased significantly. Protection still occurs over and immediately upstream of the TATA element indicating that the TBP binding is more resistant to the high salt than the downstream contacts. Assembly of an initiation complex might require similar structural changes to those seen here.

## DISCUSSION

By using an appropriate selection of DNA primers, we have been able to construct DNA fragments containing radioactive nucleotides in selected patches of the hsp70 promoter. Protein–DNA crosslinking with these selectively labeled fragments have identified polypeptides that contact specific regions of the promoter in a TATA-dependent manner. The TBP subunit of TFIID crosslinks specifically to the TATA element. Additional polypeptides were found to crosslink in the following way. A 26 kd polypeptide crosslinks to the TATA box. A 150

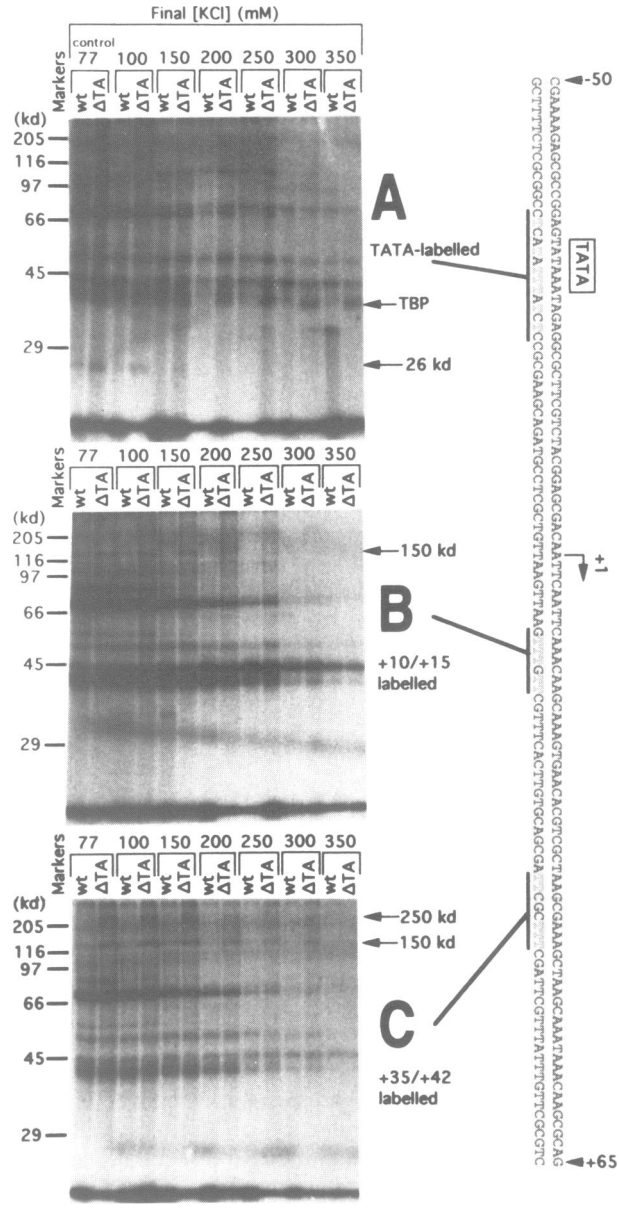


**Figure 5.** Effects of increasing salt concentration on the crosslinking of different TATA-dependent interactions. The lines alongside the sequence indicate those nucleotides incorporated during the first polymerization step, and the highlighted T's are those that included $^{32}P$. In each case, the TATA complex was formed in the presence of either the wild-type competitor or the ΔTATA competitor. The salt concentration was then changed by addition of a KCl stock. Samples were then crosslinked. (**A**) The 42 kd TBP persists in its binding to the DNA even after a challenge at 350 mM KCl; however, labeling of a 26 kd polypeptide at the TATA box is abolished by 200 mM KCl. (**B**) The efficiency of the 150 kd peptide's tagging between +10 and +15 is severely decreased above 150 mM KCl. (**C**) With label further downstream, between +35 and +42, labeling of the 150 kd is significantly reduced above 150 mM. Also, tagging of the polypeptide greater than 205 kd diminishes above 150 mM KCl.

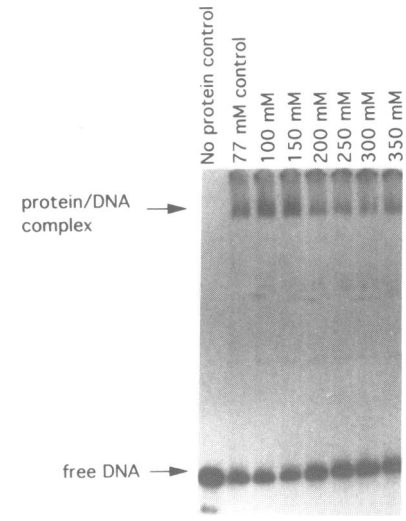


**Figure 6.** PAGE mobility shift assay of KCl-challenged protein/DNA complexes. Binding assays were performed in parallel with those shown in Fig. 6. Samples were subjected to native gel electrophoresis instead of crosslinking after the KCl challenge.

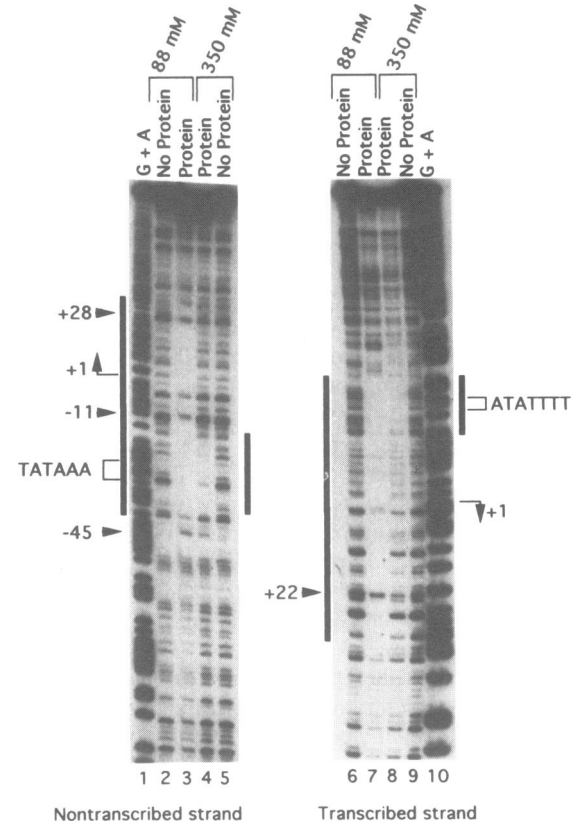


**Figure 7.** DNase I footprinting of the TATA complex in low and high salt. TATA complex was first formed on the DNA at 88 mM KCl (lanes 2, 3, 7, 8). Then, the salt was either left unchanged (lanes 2, 3, 6, and 7) or elevated to 350 mM (lanes 4, 5, 8, 9). Brief DNase I digestion was then performed. Lanes 1 and 10 are markers, showing chemical cleavage at A and G residues.

kd polypeptide crosslinks in two places downstream of the transcription start site. The first region of contact is between +10 and +15, the next region is between +35 and +47. A 250 kd polypeptide crosslinks primarily in the region from +35 to +42. As the DNase I footprint produced by the complex (Figure 5)

is almost identical to the footprint produced by immunopurified TFIID (7), it is likely that the polypeptides contacting the DNA in the region of the footprint are subunits of TFIID. Strong support for this hypothesis is provided by the observation that the polypeptides crosslinking in the region known to be contacted by TFIID match the reported sizes of subunits of TFIID (17). Further analysis, however, is required to establish definitively that these polypeptides are actually TAFs.

From the pattern of crosslinking, we can estimate the precision of the approach for mapping contacts of polypeptides. Two observations suggest that the protein must crosslink to the DNA within 10 nucleotides of the region containing the radioactivity in order to be tagged. The first observation concerns the 150 kd polypeptide. This polypeptide was tagged when the label was in the region from +10 to +15 and in the region from +35 to +42, but not when the label was in the region from +18 to +25. The second concerns the 250 kd polypeptide. This polypeptide was tagged when the label was in the region from +35 to +42 but not when the label was positioned at nucleotides +46 and +47. This latter case is a particularly good illustration of the resolution since the 150 kd polypeptide is tagged when the label is in either the +35 to +42 region or at nucleotides +46 and +47.

It is interesting to note that no TATA-dependent contacts were detected when the radiolabel was placed in the region between +18 and +25. On the contrary, the label in this position led to more tagging of nonspecific proteins than when label was placed elsewhere. Examination of the DNase I footprint of TFIID indicates that there are hypersensitive sites within the footprint at +22 on one strand and at +28 on the other strand. This 6 base pair stagger suggests that one face of the helix is accessible, and this could explain the high level of nonspecifically tagged proteins that accompanies the fragment labeled in this region. This high level of nonspecific labeling could obscure detection of the polypeptide that is contacting the opposite face of the helix, or the polypeptide on the opposite face may not crosslink efficiently.

By combining the techniques of crosslinking, DNase I footprinting and electrophoretic mobility shift assays, we have been able to identify intriguing features about the TATA-dependent contacts that occur downstream of the transcription start. The 150 kd polypeptide contacts in a region outside of the TFIID footprint that has been detected on the normal hsp70 promoter. This has puzzled us for some time, but recent data from the analysis of promoters with 3′ deletions provides a simple explanation for the discrepency between the crosslinking and footprinting data (Emanuel and Gilmour, unpublished results). These 3′ deletions place novel sequences downstream of the +35 region. When different 3′ deletions (+33, +23, or +18 described in (7)) are bound to immunopurified TFIID, the region 45 nucleotides downstream of the transcription start is found to be hypersensitivie to DNase 1. This is readily apparent because the hypersensitive sites arise in regions that are normally weakly cut by DNase 1 in naked DNA. By contrast, the normal hsp70 promoter is strongly cut in in the +45 region even when TFIID is absent and this probably obscures the detection of the TFIID interaction. For the 3′ deletions, the hypersensitive sites occur at the same distance from the start site but on different sequences. Hence the contact seems to be independent of the sequence in the +45 region.

As noted previously, the region downstream of the TATA element must accomodate both the interaction of TFIID and the interaction of RNA polymerase II. The effect of salt on the crosslinking of TATA-dependent polypeptides suggests one way by which these interactions may be integrated. Raising salt concentrations caused preferential dissociation of polypeptides from the region downstream of the TATA box while TBP remained associated with the TATA box. With the possible exception TAF 30, most of the TFIID complex appears to remain intact at salt concentrations up to 0.5M [(18) and unpublished observation]. Our gel-shift analysis is consistant with the protein−DNA complex remaining intact in high salt, although we can not rule-out the possibility that the complex rapidly reassembled when the complexes entered the gel. Under normal circumstances, the preinitiation complex could displace the downstream contacts of TFIID. The TFIID could remain tethered to the promoter by the association of the TBP subunit with the TATA element.

If the interaction of the 150 kd polypeptide in the region downstream of +35 were strong enough, it might provide the blockade that pauses the RNA polymerase II. In fact, the crosslinking indicates that the contact of the 150 kd polypeptide to the region downstream of +35 appears to be more resistant to the high salt than the contact of the 150 kd polypeptide in the +10 to +15 region. A model that describes the pausing of RNA polymerase II in the hsp70 promoter must accomodate the following pieces of information. First, there appears to be no strong sequence requirements downstream of the pausing site as RNA polymerase II still pauses on promoters that have different sequences downstream of +24 (3, 4). Second, the mechanism must be general since it occurs with other *Drosophila* genes and has been found to occur on the human c-myc gene in human cells (2, 3, 9, 6). Third, the model must explain how GAGA sequences located upstream of the TATA element and sequences located downstream of the transcription start can act in a compensatory manner to maintain significant levels of paused polymerase when mutations are introduced in one or the other of these regions (4). A common component could be TFIID. TFIID-binding depends on sequence-specific interactions from the TATA element down to approximately +30. Its interaction is also dependent on upstream activators. The GAGA sequence binds a known transcriptional activator which might in some way facilitate TFIID binding. A sequence-independent DNA contact between TAF150 and/or TAF250 downstream of +35 could then provide the block for the polymerase. Experiments are currently in progress to test this model.

The crosslinking analysis has provided intriguing new insights into several TATA-dependent interactions. Several proteins that are involved in transcribing the hsp70 promoter can be detected by crosslinking. These include RNA polymerase II (see Fig. 4), GAGA factor (19), and heat shock factor (Gilmour, unpublished observations). The protein/DNA crosslinking approach described here should allow us to study the dynamic interplay between these components as they interact with the promoter and orchestrate transcriptional initiation and elongation. In addition, the observation that these proteins crosslink to the DNA under *in vitro* conditions indicates that they could also be examined by crosslinking *in vivo* (20).

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