A DNA-binding domain of human transcription factor IIC2

Pierre A. Boulanger+1, Noelle D. L'Etoile and Arnold J. Berk*

Molecular Biology Institute and Department of Microbiology, University of California, Los Angeles, CA 90024-1570, USA

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ABSTRACT

Transcription factor IIC2 is required for in vitro transcription of the adenovirus 2 VA1 gene and binds with high affinity to its B-box promoter element which is an 18 bp perfect inverted repeat. Partial proteolysis of TFIIIC2 with chymotrypsin and Staphylococcus aureus V8 protease yielded a species which produced a discrete band in a gel shift assay with about twice the mobility of the undigested complex. Chymotrypsin-digested TFIIIC2 produced a DNase I footprint virtually identical to that of the undigested protein, but the stability of the protein-VA1 DNA complex was drastically reduced and the in vitro transcriptional activity was eliminated. These results indicate that a chymotrypsin-resistant domain of TFIIIC2 binds to the B-box sequence. We speculate that stable binding requires protease sensitive cooperative interactions between TFIIIC2 DNA-binding domains.

INTRODUCTION

Eukaryotic tRNA genes and the adenovirus genes encoding the two viral-associated RNAs (VA1 and VA2 RNAs) contain internal transcription control regions consisting of two non-contiguous DNA segments termed the A and B boxes (1–4). These genes are transcribed by RNA polymerase III (pol III) in conjunction with pol III specific transcription factors (5). One of these factors, TFIIIC, has been shown to bind to the intragenic promoter regions and form a stable complex which is activated for transcription by the addition of pol III and TFIIIB (6–11).

Unlike yeast TFIIIC (12), TFIIIC from higher eukaryotes can be separated into two components required for in vitro transcription (13–15). We have referred to these as TFIIIC1 and TFIIIC2 in preparations from human cells. TFIIIC2, binds to the B-box promoter element of tRNA and the adenovirus type 2 VA1 RNA genes (13,15) and TFIIIC1 extends the footprint over the A-box region (13). The Ad2 VA1 B-box is contained in an eighteen basepair perfect inverted repeat (1). TFIIIC2 binds to this region with very high affinity (K₈ = 2×10¹¹ M⁻¹) and produces a DNase I footprint centered over the inverted repeat (16). TFIIIC2 behaves as a high molecular weight protein with a sedimentation coefficient of 18S corresponding to a globular protein of 400–500 kDa (13). Highly purified TFIIIC2 contains five polypeptides ranging in size from 60 to 230 kDa (17). The largest of these can be specifically UV cross-linked to the VA1 B-box sequence (16,17). Yeast TFIIIC (called τ) is also a large protein composed of several polypeptide chains (12). However, unlike TFIIIC2, τ protects both the A-box and B-box promoter elements of tRNA genes from DNase I digestion (9,18), and two polypeptides can be UV cross-linked to DNA (12). The larger of these binds specifically to a tRNA gene sequence when bound to nitrocellulose (19). Thus yeast τ appears to contain a combination of the human TFIIIC1 plus TFIIIC2 activities.
Limited proteolytic digestion has been a useful procedure for defining functional domains in proteins. This technique has proven to be particularly rewarding when a single or limited number of protease-sensitive regions of a protein separate protease-resistant domains. This is the case for DNA-binding proteins such as the phage lambda repressor (20), the yeast α2 repressor (21), the adenovirus single-stranded DNA binding protein (22), the Xenopus transcription factor TFIII A (23), and more recently, the yeast transcription factor τ (24). Proteolysis of τ suggests the existence of two functional domains in the high molecular weight protein: a τ-A domain which interacts with A-box sequence and is sensitive to proteolysis, and a τ-B domain which binds the B-box sequence and is resistant to protease digestion (24). The TFIIIC2 protein which we studied earlier had properties similar to the τ-B domain of the yeast protein in that it interacts with the B-box but not the A-box promoter element. Consequently we analyzed the properties of partially proteolyzed TFIIIC2 in a study analogous to the one performed with yeast τ. We found that a chymotrypsin-resistant fragment of TFIIIC2 yields a DNase I footprint virtually identical to that of the intact TFIIIC2 providing evidence for a DNA binding domain. Regions of TFIIIC2 removed by partial proteolysis were found to be essential for forming stable DNA-protein complexes with VA1 DNA and for complementation with TFIIIC1, TFIIIB and pol III for in vitro transcription of VA1 RNA.

MATERIALS AND METHODS

Purification of TFIIIC2
TFIIIC2 was prepared from 293 cells grown in suspension culture in spinner MEM with 5% newborn calf serum and antibiotics. TFIIIC2 used in figures 1 and 2 was purified from S-100 extracts by chromatography on PC-11 phosphocellulose and FPLC Mono Q as described (13). TFIIIC2 used in figures 3 and 4 was purified from nuclear extracts by ammonium sulfate precipitation, chromatography on S-300 and affinity chromatography on a column of multimerized B-box DNA sequence as described (17).

In vitro transcription
In vitro transcription was performed as described (25) using TFIIIC1 purified through Mono Q chromatography and fraction B containing TFIIIB and pol III purified by gradient elution from phosphocellulose as described (13). The 40 μl reactions were incubated for 60 min at 30°C and contained 0.6 μg TFIIIC1 fraction protein, 1.3 μg fraction B protein, 0.35 μg pVA1, 0.15 μg pUC18 and the indicated amounts of TFIIIC2 or chymotrypsin-digested TFIIIC2. In vitro transcribed RNA was extracted and analyzed by gel electrophoresis and autoradiography as described (25).

DNase I footprinting
DNase I footprinting was performed in solution and analyzed directly on sequencing gels as described (13). Alternatively, free and protein-bound probe were separated following DNase I digestion by electrophoresis in low percentage polyacrylamide gels (as in the gel binding assay), DNA was recovered and subjected to analysis on sequencing gels as described (16).

Gel binding assay
Binding of TFIIIC2 or proteolyzed TFIIIC2 to VA1 DNA was analyzed by the gel retardation assay as described (16). Binding reactions were in 10 μl of 75 mM KCl, 0.5 mM Na₂EDTA, 2.5 mM MgCl₂, 5 mM DTT, 5% glycerol, 0.1% NP₄₀. 0.1 to 1 ng end-labeled 247 bp VA1 probe (5,000 to 15,000 Cerenkov cpm, prepared as described, 16) was incubated with 0.5 to 5 μg protein in the presence of 0.05 to 0.5 μg poly(dI:dC)-
Figure 1. Gel retardation assay of undigested (lanes 1–4) and proteolyzed (lanes 5–14) TFIIIC2. Aliquots of \(^{32}\)P-labeled VA1 DNA probe were incubated with increasing concentrations of TFIIIC2. Lane 1: no protein added; lane 2: 1 \(\mu\)g; lane 3: 2 \(\mu\)g; lanes 4–14: 3 \(\mu\)g. After 30 min binding at 25°C, chymotrypsin (lanes 5–9) or \(S\). aureus V8 protease (lanes 10–14) was added to the incubation mixtures, and hydrolysis allowed to proceed for 10 min at 25°C. Enzyme concentrations were: 0 (lanes 5,10), 1 ng (lanes 6,11), 5 ng (lanes 7,12), 10 ng (lanes 8,13), and 25 ng (lanes 9,14) per 10 \(\mu\)l sample. Band I corresponds to free DNA probe, band III to the specific retarded complex formed between TFIIIC2 and VA1 DNA, and band II to the proteolyzed TFIIIC2-VA1 complex. The minor retarded band indicated by the black dot corresponds to a protein factor binding outside of the A- and B-box control regions (16).

poly(dI.dC) for 30 min at 25°C. Samples were loaded on a 3.5% polyacrylamide gel (acrylamide:bisacrylamide ratio of 50:1.5) cast in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA. Prerun gels were loaded while current was on and the buffer was stirred and recirculated during electrophoresis (16) at 110 V (20–30 mA) for 3 hours at room temperature.

Enzymes and enzymatic proteolysis

Chymotrypsin (TLCK-treated), trypsin (TPCK-treated) and \(S\). aureus V8 protease were purchased from Sigma. They were used at concentrations of 1, 5, 10, 25, and 50 ng of enzyme per sample. 1 \(\mu\)l protease solution was added to 10 \(\mu\)l of DNA binding reactions with TFIIIC2 and incubated at 25°C for 10 min. Hydrolysis was arrested with 0.25 \(\mu\)g phenylmethysulfonyl fluoride (Sigma) in the case of trypsin, or 0.25 \(\mu\)g chymostatin (Sigma) in the case of chymotrypsin.

RESULTS

Proteolytic digestion of TFIIIC2-VA1 complex

TFIIIC2 fractions were incubated with a 127 bp fragment from the VA1 gene including the A- and B-box promoter elements. Trypsin, chymotrypsin, or \(S\). aureus V8 (Staph. V8) proteases were then added in increasing amounts to the reactions and the DNA-protein complexes were resolved by electrophoresis on a 3.5% polyacrylamide gel.
Figure 2. DNase I footprint analysis of the VAl gene with undigested and chymotrypsin-digested TFIIIC2. (A) Increasing amounts of undigested TFIIIC2 (5, 10, 15 and 20 μg) were added to reaction mixtures shown in lanes 5–8. Constant amounts of TFIIIC2 (20 μg) and increasing amounts of chymotrypsin (1, 5, 10, and 25 ng) were assayed in lanes 9–12. The presence or absence of chymotrypsin (CT) is indicated by (+) or (−), respectively, at the top of the figure. Chymotrypsin digestion (10 min at 25°C) was arrested by chymostatin prior to DNase I digestion. An end-labeled Msp I digest of pBR322 was used as marker (lane 1). Lanes 2, 3: native and denatured VAl DNA probe, respectively. Lanes 4, 13: control samples, no protein added. (B) DNase I footprinting of
The mobility of the DNA-protein complex was dramatically altered by proteolytic digestion (fig. 1). The specific retarded band of TFIIIC2-VA1 DNA complex (band III) disappeared progressively with increasing protease concentration. In the case of chymotrypsin digestion, the slow migrating complex of band III was replaced by a discretely-migrating complex of higher mobility designated band II at 10 and 25 ng chymotrypsin (lanes 8 and 9). The amount of probe retarded in the chymotrypsin-digested complex II was diminished compared to the amount bound by undigested TFIIIC2. An intermediate complex with mobility between that of band II and band III was observed with 5 ng chymotrypsin (lane 7).

Digestion with staph. V8 protease produced a DNA-protein complex of similar mobility to chymotrypsin-digested complex II (lanes 10–14). Trypsin digestion resulted in the progressive loss of the TFIIIC2-VA1 complex band III without the occurrence of any discrete band of higher mobility (data not shown). The sharpness of chymotrypsin-digested band II suggested the existence of a fairly homogeneous, partially digested TFIIIC2-VA1 complex. For this reason, and because chymotrypsin hydrolysis is readily controlled with the specific inhibitor chymostatin, chymotrypsin was the protease used in further studies. The same retarded band pattern was obtained when TFIIIC2 was incubated with chymotrypsin and further proteolysis inhibited with chymostatin prior to incubation with VA1 DNA (see fig. 3).

Chymotrypsin-cleaved TFIIIC2 still binds to the B-box region of the VA1 gene
For a DNA-protein complex migrating in a neutral gel, the majority of the electric charge is carried by the DNA and the protein charge does not greatly influence its migration. Consequently, the much greater mobility of the TFIIIC2-VA1 DNA complex most likely corresponds to a substantial loss of protein mass from the preformed complex. By analogy with other DNA binding proteins, it appeared likely that chymotrypsin digestion had released a DNA-binding domain. To test whether the chymotrypsin-digested TFIIIC2 bound to VA1 DNA similarly to undigested TFIIIC2, we analyzed the DNase I footprint produced by the two protein fractions.

TFIIIC2 protects a 40 bp region of the VA1 DNA centered over the B-box, from nucleotide +42 to +82 from the start of transcription (13). The same protected region occurred in DNA present in the retarded complex obtained in a gel shift assay and extracted from the gel (16). In a first set of experiments, aliquots of VA1 DNA-TFIIIC2 complexes were incubated with increasing amounts of chymotrypsin. Chymotrypsin digestion was arrested by addition of chymostatin and DNase I was added under the conditions of DNase I footprinting, and the products resolved on a sequencing gel (fig. 2A). No significant change in the pattern of B-box protection was observed following chymotrypsin digestion under conditions which converted all of the TFIIIC2 to the faster migrating complex II (lanes 11 and 12).

In a second type of experiment, TFIIIC2 was incubated with the same end-labeled VA1 DNA probe and digested with chymotrypsin. Following chymotrypsin digestion the samples were digested with DNase I and analyzed by the gel retardation assay. Free probe and chymotrypsin-digested TFIIIC2-VA1 DNA complex isolated from a retardation gel. 5 μg aliquots of TFIIIC2 digested with 25 ng of chymotrypsin were incubated with VA1 DNA probe as in (A) and the incubation mixtures were treated with 6 ng (lanes 2,3) or 3 ng (lanes 4,5) of DNase I. The resulting complexes were analyzed by gel shift assay electrophoresis, as depicted in fig.1. Free migrating probe (lanes 2,5) and the complex of digested TFIIIC2-VA1 DNA present in retarded band II (lanes 3,4) were extracted and analyzed on an 8%-8M urea polyacrylamide gel. Lane 1: pBR322 Msp I markers. A schematic representation of the VA1 gene with the protected region is diagramed on the right of both panels.
Figure 3. In vitro transcription using chymotrypsin-digested TFIIIC2. (A) Affinity purified TFIIIC2 was digested with chymotrypsin as described in Materials and Methods. 0.5 μl of undigested TFIIIC2 (C2) retarded an equivalent amount of VA1 probe as 4 μg of chymotrypsin digested TFIIIC2 (CTC2). (B) In vitro transcription with undigested and chymotrypsin-digested TFIIIC2. Equivalent gel retarding activities of TFIIIC2 and chymotrypsin-digested TFIIIC2 (aliquots of the same preparations analyzed in (A)) were used in in vitro transcription reactions. The chymotrypsin digestion was terminated by addition of chymostatin before addition to the transcription reaction (CTC2). In the control reaction (C2), the same amounts of chymotrypsin and chymostatin used to prepare CTC2 were premixed and added to undigested TFIIIC2 and incubated before addition to the transcription reaction. The lane marked (−) shows a transcription reaction without added TFIIIC2. VA1 shows the position of VA1 RNA.

Chymotrypsin-digested TFIIIC2 band II were visualized by autoradiography of the gel, the complexes were eluted from gel slices, and the isolated DNA was denatured and analyzed on a sequencing gel (fig.2B). The DNase I footprint associated with the chymotrypsin-digested TFIIIC2 complex (lanes 3 and 4) was centered over the B-box and was very similar to the DNase I footprint observed with undigested TFIIIC2 in solution (fig. 2A, lanes 6–8). Thus, in spite of the drastic change in the mobility of the chymotrypsin-digested TFIIIC2-VA1 DNA complex, there was little change in the DNase I footprint produced with the chymotrypsin-digested complex.

**Chymotrypsin-digested TFIIIC2 is inactive for in vitro transcription**

To determine whether chymotrypsin-digested TFIIIC2 was still transcriptionally active, TFIIIC2 was assayed in an in vitro reconstituted transcription reaction with TFIIIC1, TFIIIB and pol III (fig. 3). Equivalent gel retarding activity of undigested and chymotrypsin-digested TFIIIC2 (fig. 3A, 0.5 μl and 4 μl, respectively) were used in the transcription reactions shown in fig. 3B. In this preparation of chymotrypsin-digested TFIIIC2, a DNA-protein complex with more rapid migration than complex II was also observed. However, this complex could not be specifically competed with B-box DNA (data not shown), and therefore represented a non-specific binding activity generated by chymotrypsin digestion. The chymotrypsin-digested TFIIIC2 did not support in vitro transcription (fig. 3B, middle...
Figure 4. Off rates of undigested and chymotrypsin-digested TFIIIC2. Untreated (A) and chymotrypsin-digested (B) TFIIIC2 were incubated with 1.5 fmole VAI probe for 60 min at 25°C. 150 fmole of ligated B-box oligonucleotide (15) were added to the binding reaction at time 0. 10 µl aliquots were removed from the binding reaction at the indicated times after addition of competitor and loaded onto a running, native gel. Autoradiograms of the gels are shown in the upper panels. The autoradiograms were quantitated by scanning densitometry. The areas under the specific retarded complex bands were divided by the sum of the areas under all peaks for each time point and the ln of this fraction was plotted in the bottom panels.

A control was performed to show that the inhibition of transcription was not due to a failure of chymostatin to inhibit chymotrypsin digestion. The chymotrypsin-digested TFIIIC2 (CTC2) used in the gel retardation (fig. 3A) and transcription (fig. 3B) assays was prepared by incubation with chymotrypsin for 15 min followed by addition of chymostatin. The same amounts of chymotrypsin and chymostatin used to prepare chymotrypsin-digested TFIIIC2 were premixed and incubated with TFIIIC2 for 15 min before addition to the reconstituted in vitro transcription reaction shown in fig. 3B, lane C2. Thus, inhibition of chymotrypsin by chymostatin before digestion of TFIIIC2 prevented both formation of the high mobility complex assayed by gel retardation and inhibition of transcription. The right lane of figure 3B (-) shows a transcription reaction with TFIIIC1, TFIIIB and pol III, but no added TFIIIC2.
Chymotrypsin digestion greatly reduces the stability of the DNA-protein complex

To test the effect of chymotrypsin digestion on the stability of the TFIIIC2-DNA complex, we measured the off-time of TFIIIC2 from VA1 DNA following challenge with a large molar excess of B-box DNA. TFIIIC2 (fig.4A) or chymotrypsin-digested TFIIIC2 (fig.4B) was incubated with 1.5 fmol of labeled VA1 probe for 60 min at 25°C and 150 fmol of multimerized B-box synthetic DNA was added at time zero. Aliquots were removed from the reaction at various times after addition of competitor and loaded onto a running, low percentage polyacrylamide gel. An autoradiogram of the resulting gels is shown on the top of figure 4. The autoradiograms were quantitated by densitometry and the In of the fraction of probe retarded in the TFIIIC2 or chymotrypsin-digested TFIIIC2 complexes were calculated and plotted in the bottom of figure 4. The TFIIIC2-VA1 DNA complex was fairly stable under these conditions (25°C, 100 mM KCl), dissociating with first order kinetics with an average half-time measured in several similar experiments of 40 min. In contrast, the chymotrypsin-digested TFIIIC2 complex was much less stable to competition with B-box DNA, with a dissociation half-time of less than 0.5 min.

DISCUSSION

Partially proteolyzed transcription factor IIIC2 formed a specific DNA-protein complex with the VA1 B-box promoter element which had much greater mobility in a low percentage polyacrylamide gel than the undigested TFIIIC2-DNA complex (fig.1). Nonetheless, proteolyzed TFIIIC2 generated a DNase I footprint very similar to the footprint generated with undigested TFIIIC2 (fig.2). The much greater mobility of the digested DNA-protein complex suggests that a substantial proportion of the protein mass was removed from TFIIIC2 by partial proteolysis. Therefore, partial proteolysis resulted in the cleavage of a DNA-binding domain from the remainder of the transcription factor. We attempted to determine the size of the protease resistant DNA binding polypeptide by UV cross-linking to 32P-labeled B-box DNA. However, we were unable to detect a specifically cross-linked species even though the 230 kDa polypeptide of undigested TFIIIC2 was readily cross-linked in parallel reactions, as observed previously (16,17). The inability to cross-link chymotrypsin digested TFIIIC2 was probably a result of its greatly decreased affinity.

Similar studies have been performed with the well characterized lambda-repressor (21) and yeast α2 repressor (22) proteins. These proteins bind as dimers of a single polypeptide chain. Each subunit binds to symmetrical half-sites in the operator sequence. In both of these cases, the affinity of the isolated DNA binding domain for operator sites is greatly decreased compared to the affinity of the intact repressors (21,22). The decreased affinity of the isolated DNA binding domains results from a loss in cooperativity of binding to the two half-sites caused by separation of the binding domains (21,22).

The affinity of partially proteolyzed TFIIIC2 for B-box sequence was also greatly diminished compared to the undigested protein. This was demonstrated by the much faster off-time of partially proteolyzed TFIIIC2 from a VA1 probe when the complex was challenged with a hundred fold excess of unlabeled B-box binding sites (fig.4). Another similarity to the situation with the lambda and α2 repressors is that the TFIIIC2 binding site, the B-box, is an inverted repeat. In the case of the VA1 gene B-box it is a perfect inverted repeat of eighteen basepairs (1). The consensus B-box from tRNA genes (5) also has an inverted repeat character. By analogy to the well studied lambda and α2 repressors and operators, we speculate that TFIIIC2 binds to the two halves of the B-box region through symmetrical DNA binding domains which are separated by partial proteolysis.
Chymotrypsin-digested TFIIIC2 is inactivated for in vitro transcriptional activity. It seems likely that portions of the TFIIIC2 protein required for interacting with RNA polymerase III and the other transcription factors required for transcription, TFIIIC1 and TFIIIB, are removed or inactivated by protease digestion. However, it also possible that the much lower stability of the chymotrypsin-digested TFIIIC2 DNA-protein complex compared to the undigested DNA-protein complex also contributes to the loss of transcriptional activity.

Marzouki et al (24) performed similar partial proteolysis studies of the yeast factor τ. τ protein appears to be a more complex protein than TFIIIC2 in that it protects both the A-box and B-box of tRNA genes from DNase I digestion. Partially proteolyzed τ produced a specific DNA-protein complex with a tRNA gene which had much greater mobility in a low percentage polyacrylamide gel than the undigested τ-DNA complex, quite similar to the situation with TFIIIC2. The partially proteolyzed τ protein retained the ability to protect the B-box promoter element of a tRNA gene from DNase I digestion but lost the ability to protect the A-box region. The stability of the partially proteolyzed τ-B-box DNA complex was not analyzed. Partially proteolyzed τ also lost in vitro transcriptional activity.

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*To whom correspondence should be addressed

†Present address: Laboratoire de Virologie et Pathogenese Moleculaires, Institut de Biologie, Faculté de Medecine, 34060 Montpellier, France

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