Two forms of transcription factor TFIIC in extracts from HeLa cells

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ABSTRACT

Partially-purified preparations of transcription factor TFIIC derived from either cytoplasmic (S100) or nuclear (NE) HeLa cell extracts possessed comparable activities when assayed by in vitro transcription, although it was possible to detect sequence-specific binding to the VAI RNA gene promoter by DNase I footprinting only with TFIIC (NE). On the basis of mixing experiments, this difference could not be ascribed to either transcriptional or DNA-binding inhibitors. These findings provide further evidence for the existence of two forms of TFIIC: one which is present in both extracts and is transcriptionally active; another which predominates in nuclear extracts and is transcriptionally inactive.

INTRODUCTION

Transcription factor TFIIC was initially defined as a chromatographic fraction derived from human cell-free extracts which is required for the reconstitution of accurate transcription from all class III genes *in vitro* (1,2). Through template competition experiments, TFIIC has been shown to interact directly with the VAI and tRNA^{MET}_I genes (3). A protein present in this fraction has been shown by various cleavage interference methods to bind specifically to the internal promoters of both genes (3-6). It has been presumed that the same protein is responsible for both the observed DNA binding and transcriptional activities, though recent data demonstrating differences in TFIIC behavior (salt stability of TFIIC-VAI gene complexes (5), chromatographic separation of DNA binding and transcription activities (6)) cast some doubt on this assumption.

In this manuscript we have chosen to illustrate the apparent discrepancy between the DNA binding and transcriptional activities of TFIIC by investigating these properties in chromatographic fractions derived from two types of HeLa cell extracts, either cytoplasmic (S100) or nuclear (NE) in origin (7). DNA binding was investigated by DNase I footprinting; transcriptional activity was assayed by *in vitro* reconstitutions. The VAI RNA gene of adenovirus-2 was employed in both assays. Our results indicate that

while S100- and NE-derived fractions exhibit comparable transcriptional activities, we have been able to observe footprints only with TFIIIC (NE). Mixing experiments provided no indications of DNA binding or transcription inhibitors in either fraction. We conclude that this differential behavior is evidence for the existence of two forms of TFIIIC: one which confers transcriptional activity, another which predominates in HeLa cell nuclear extracts and is itself only capable of DNA binding.

METHODS

Protein fractions

Cytoplasmic (S100) and nuclear (NE) extracts were prepared from HeLa cells according to the Dignam protocol (7). TFIIIC-containing fractions were partially purified by chromatography over phosphocellulose as previously described (6). The TFIIIC fraction derived from a S100 eluted at 500 mM KCl and contained 3.65 mg/ml protein. The TFIIIC fraction derived from a NE eluted at 525 mM KCl and contained 1.47 mg/ml protein. TFIIIC (S100) was contaminated with trace amounts of TFIIIB and RNA polymerase III, while TFIIIC (NE) was essentially free of both these activities. The preparation of partially purified TFIIIB and RNA polymerase III used in transcription reconstitutions has been previously described (6).

DNA plasmids

Plasmid pVAI, containing the VAI RNA gene from adenovirus type 2, was constructed by ligating the 231 bp XbaI/BalI restriction fragment from plasmid pAd2-wt into a XbaI/SmaI cut pUC12 vector and transforming *E. coli* strain JM101. Plasmids were propagated and purified using standard protocols (8).

In vitro transcription

Transcription reactions (30 μ l) were performed in a buffer containing 20 mM HEPES (pH 7.9); 8% (v/v) glycerol; 3.5 mM $MgCl_2$; 70 mM KCl; 2 mM DTT; 0.6 mM each ATP, CTP, and UTP; 25 μ M [α - 32 P] GTP (4 Ci/mole); and 0.4 μ g pVAI. Proteins present in each reaction included RNA polymerase III (0.20 μ g, 29 poly(dA-dT)units (9)), TFIIIB (0.14 μ g, 5.8 VA units (10)), 7 μ g BSA, and amounts of TFIIIC as described in the figure legends. Transcription reactions were incubated at 30°C for 60 min and processed as previously described (6). Quantitation of RNA synthesis was performed by scintillation counting of the excised radioactive VAI RNA band.

DNase I footprinting

A singly 3' end labeled 261 bp DNA restriction fragment containing the VAI gene was prepared by digesting plasmid pVAI with HindIII, radiolabeling with [α - 32 P] dATP and Klenow, and cleaving with EcoRI. Footprinting reactions

were performed in a buffer containing 20 mM HEPES (pH 7.9), 8% (v/v) glycerol, 70 mM KCl, 3.5 mM MgCl₂, 2 mM DTT, 100 ng pUC12, approximately 2 ng (>10,000 cpm) labeled DNA fragment, and amounts of TFIIC as described in the figure legends. DNase I cleavage, gel electrophoresis, autoradiography, and densitometry were performed as previously described (6). TFIIC footprinting was quantitated by comparing the intensities of DNase I cleavages (indicated by arrows in figure 1) against those in the control reaction. The protection observed with 2.5 μ l TFIIC (NE) was arbitrarily chosen as 100%.

RESULTS

S100- and NE-derived TFIIC exhibit similar transcription activities

Figure 1a illustrates the amount of VAI RNA synthesized *in vitro* as a function of TFIIC concentration. Quantitation of these results is shown in Figure 1b. Both preparations of TFIIC exhibited similar transcription activities, as indicated by their near equivalence in RNA synthesis/ μ l TFIIC during the linear response segment of the titration (0-1.5 μ l TFIIC). The observed difference in RNA synthesis at saturating concentrations (>1.5 μ l) of TFIIC is believed to result from the trace quantities of complementary transcription factors present in the S100-derived fraction (see also Methods). These data indicate that the pair of TFIIC-containing fractions derived respectively from either cytoplasmic and nuclear extracts exhibit comparable transcription activities in an *in vitro* reconstituted assay.

S100- and NE-derived TFIIC exhibit very different levels of sequence-specific DNA binding activities

The specific binding of TFIIC to the internal promoter of the VAI RNA gene was analyzed by quantitative footprinting. Figure 2a shows an autoradiogram of the DNase I cleavage patterns observed in the presence of various concentrations of the two TFIIC-containing fractions. Quantitation of the DNase I cleavage protection over the B block region of the VAI gene, as determined by densitometry, is presented graphically in Figure 2b. The well-characterized TFIIC footprint, centered on the B block consensus element within the internal promoter of the VAI gene, was only observed with increasing concentrations of TFIIC (NE) (4-6). The slight protection observed with high concentrations of TFIIC (S100) is thought to result from non-specific protein/DNA interactions nucleating from the end of the DNA. The relationship between DNase I cleavage protection over the B block of the VAI gene and the concentration of TFIIC (NE) correlates well with a binding equilibrium near saturation (Figure 2b). By contrast, no detectable interaction with the VAI gene internal promoter could be detected when

Figure 1:

Titration of TFIIIC transcriptional activity. *In vitro* transcription reactions were performed using plasmid pVAI as a template as described in the Methods. (A) Autoradiogram of transcription products. VAI RNA is indicated by an arrow. The amount of either TFIIIC (S100) or TFIIIC (NE) present in each reaction is indicated above each lane. (B) Quantitation of transcription. VAI RNA synthesis is in pmoles RNA. Closed circles are for TFIIIC (NE); open circles are for TFIIIC (S100).

comparable concentrations of TFIIIC (S100) were present. In fact, further purification and concentration of TFIIIC (S100) have not yet yielded a protein fraction with which we are able to detect footprints (data not shown). This failure to observe footprints does not imply that transcriptionally active

Figure 2:

Titration of TFIIIC sequence-specific DNA binding activity. DNase I footprinting reactions were performed with a singly 3' end labeled DNA fragment containing the VAI RNA gene as described in the Methods. (A) Autoradiogram of DNase I cleavage products. The amount of either TFIIIC (S100) or TFIIIC (NE) present in each reaction is indicated above each lane. DNase I cleavages used in quantifying protection are indicated by arrows. (B) Quantitation of TFIIIC binding. Quantitation of DNase I cleavage protection provided by TFIIIC was performed as described in the Methods. Closed circles are for TFIIIC (NE); open circles are for TFIIIC (S100).

TFIIIC is incapable of sequence specific binding to the VAI gene promoter, but rather reflects the technical limitations of DNase I footprinting with fractions containing an excess of nonspecific DNA binding proteins.

Figure 3:

Investigation of transcription or DNA binding inhibitors by mixing experiments. (A) Quantitation of transcription as a function of TFIIC (S100) and TFIIC (NE) present. VAI RNA synthesis is in pmoles RNA. (B) Quantitation of the effect of increasing amounts of TFIIC (S100) on the ability of a fixed quantity of TFIIC (NE) to footprint. 0.75 µl TFIIC (NE) was present in each reaction.

Mixing experiments exclude the possibility of transcription or DNA binding inhibitors

Since only partially purified preparations of TFIIC were employed in the above analyses, the possibility remained that the observed discrepancy between transcription and DNA binding activities could result from the presence of inhibitors. Thus, either TFIIC (S100) could contain an inhibitor of specific DNA binding, or TFIIC (NE) could contain an inhibitor of transcription. The existence of the presumptive inhibitors was investigated by a series of mixing experiments. Figure 3a shows the effect of different ratios of TFIIC (S100) and TFIIC (NE) on VAI RNA synthesis. Intermediate ratios of S100- and NE-derived TFIIC demonstrated a slight enhancement of transcription, rather than the depression of transcription expected if a transcription inhibitor was present. Figure 3b shows the effect of increasing concentrations of TFIIC (S100) on the ability of a minimal quantity of TFIIC (NE) to interact with the VAI gene promoter as demonstrated by DNase I footprinting. Increasing amounts of TFIIC (S100) did not inhibit footprinting, but actually improved protection slightly. This effect may simply be the result of a stabilization of TFIIC due to the increase in overall protein concentration. Thus, with the mixing experiments excluding the possibility of artifacts due to inhibitors, the observed discrepancy in the DNA binding abilities of two TFIIC preparations possessing similar

transcriptional activities would appear to result from properties which are intrinsic to TFIIIC.

DISCUSSION

Evidence for two forms of the transcription factor TFIIIC stem from the observed differences in its behavior when analyzed by functional assays (e.g. *in vitro* transcription reconstitution) versus physical methods (e.g. DNase I footprinting). These differences include such phenomena as an increased stability to high ionic strengths of the transcriptionally active TFIIIC:VAI gene complex (5), and a chromatographic separation of transcription and footprinting activities (6). In this paper we have provided further evidence for two forms of TFIIIC by demonstrating that two preparations of TFIIIC having comparable transcription activities contain vastly different amounts of a protein capable of binding specifically to the internal promoter (B-block consensus element) of the VAI gene.

What could account for the observed differences in TFIIIC behavior? First it must be remembered that the two most commonly used methods of analyzing TFIIIC activity, *in vitro* transcription and DNase I footprinting, exhibit very different maximal sensitivities. Cleavage protection methods such as DNase I footprinting require that a majority of binding sites are occupied in order for a protection to be observed. The detection limit of DNase I footprinting is therefore restricted by the specificity of the protein-DNA interaction, the equilibrium binding constant, and the specific activity of the radiolabeled DNA fragment. Typically, a minimum of 1 fmole protein is required. *In vitro* transcription, however, does not require the occupation of all potential templates with transcription factors. In fact, if multiple rounds of transcription are taking place (as has been observed with 5S rRNA gene transcription *in vitro* (11)), the sensitivity of this assay as a function of active TFIIIC would increase accordingly. Detection limits several orders of magnitude less than those achieved with physical methods such as footprinting are therefore possible with an enzymatic assay like *in vitro* transcription. Thus it is easily conceivable that the less sensitive DNA binding assay might miss a minor component which is actually responsible for functional activity. Earlier we had hypothesized that two populations of B-block binding proteins were present in HeLa nuclear extracts: a predominant form which is inactive in transcription and a minor form which is active (6). Physical differences between these two forms could, in conjunction with the different sensitivities inherent in the two methods of analysis, account for the aforementioned discrepancies in TFIIIC behavior.

The choice of comparing TFIIIC derived from different cellular extracts (cytoplasmic and nuclear) was based in part on their widespread use in investigating class III gene transcription *in vitro*. Initially, cytoplasmic extracts prepared by the protocol of Weil et al. (10) (150 mM KCl final concentration) were employed since they contained transcription factors TFIIIA, TFIIIB, TFIIIC, and most of the RNA polymerase III activity. Unfortunately, TFIIIC derived from these extracts has exhibited a variable ability to be analyzed by footprinting assays, usually requiring multiple chromatographic steps in order to produce a sufficiently concentrated sample for this purpose (4,5). TFIIIC has also been shown to be present in nuclear extracts prepared by the method of Dignam et al. (7) (>200 mM KCl final concentration). TFIIIC derived from this source readily footprints after only a single chromatographic fractionation (6). It is interesting to note that the complementary cytoplasmic extracts prepared at low ionic strengths (Dignam protocol, 10 mM KCl), while active in transcription, were consistently unable to afford suitable TFIIIC for footprinting even after further chromatography and concentration. The above observations leads us to conclude that the ionic strength present at the time of extraction is critical for obtaining TFIIIC of sufficient concentration for such purposes as footprinting.

It is worthwhile to speculate on the possible causes for the observed two forms of TFIIIC, and their potential ramifications on future studies of these and other transcription factors. One possibility is that the transcriptionally inactive form of TFIIIC is an experimental artifact of extract preparation resulting, for example, from partial proteolysis or dephosphorylation. These results would thus be of considerable practical importance both with respect to the choice of the extract used in functional studies of TFIIIC and with respect to the increasing number of other studies in which various factor-DNA interactions are analyzed without concomitant functional (transcription) studies. A second and more interesting possibility is that both forms of TFIIIC exist *in vivo*, thus indicating a general mechanism of class III gene regulation through the interconversion of active and inactive forms. A resolution of these two possibilities requires purification of transcription factor TFIIIC. Further work is currently under way in our laboratory.

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