

Transcriptional activation assays of recombinant transcription factors

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Transcriptional activation assays provide key functional tests of the activity of recombinant transcription factors. Nuclear extracts lacking the specific factor to be tested are obtained by antibody depletion, by heat treatment, or from naturally depleted sources. In this report, we describe transcriptional activation assays which have been developed for quality assurance testing of SP1 and TFIID (TATA-binding protein).

Introduction

The transcription factors which control the specificity and rate of mRNA synthesis by RNA polymerase II *in vitro* have emerged as highly modular proteins containing remarkably separable domains of DNA binding activity and transcriptional enhancement (1-3). Both *in vitro* and *in vivo* functional studies have demonstrated that the seemingly independent domains can be exchanged among various factors (4-6).

These independent domains pose a problem for investigators designing *in vitro* transcription experiments with factors expressed from cDNAs. While it is relatively straightforward to test the integrity of the DNA binding domain by gel shift or footprinting assays, it is difficult to test the transcriptional activation potential of a recombinant factor. If the DNA binding domain of a recombinant factor recognizes its specific DNA binding site, the activation domain *cannot* be assumed to function as well.

Reconstitution or antibody depletion

The experimental strategies used to demonstrate transcriptional activation *in vitro* fall into three broad classes. First is the classic biochemical approach of reconstitution. This painstaking method was used to identify the basal, or general, transcription factors for the first time from HeLa extracts (7-9) and subsequently from other systems, such as *Drosophila* (10). The goal of reconstitution is to trace a particular transcriptional activity as it is being purified from a crude extract.

In an analogous approach, crude extracts may be depleted of a specific factor by immunoaffinity chromatography and reconstituted with a purified component. This method suffers from the inability of the antibody to remove *only* the individual target protein with a high degree of certainty. Artifacts protein-protein associations may take place in an extract, and the antibody against one protein may inadvertently remove another essential protein from the extract. While detergents could be employed to

reduce complex formation in the presence of the antibody, this may inhibit the activity of the depleted extract.

Complementation using naturally deficient extracts

A second approach takes advantage of "null background" nuclear extracts isolated from cell types naturally lacking a particular transcription factor activity. In the yeast *S. cerevisiae*, strains genetically engineered to be deficient for a given activity provide the background for biochemical complementation *in vitro* (11 and references therein).

In mammalian cells, the embryonal carcinoma cell line P19, if undifferentiated, expresses the c-jun component of the AP1 transcriptional activity at very low levels (12), making it an attractive system to test the properties of recombinant c-jun *in vitro* or *in vivo*.

Similarly, *Drosophila* cells provide a neutral background for, among other factors, mammalian glucocorticoid receptors, whose transcriptional activity may be assayed without interference by any homologous endogenous factor (13).

Complementation of heat-treated HeLa nuclear extracts

Roeder and colleagues (14) have used the selective heat lability of TFIID to create TFIID-depleted nuclear extracts. Complementation assays were then used to facilitate the screening of chromatographic fractions of HeLa TFIID as a substitute for the traditional screening method of reconstitution. (See the article on [page 10](#) for a review of TFIID.) This method has been extended to the TFIID activity of the *Drosophila* system (cited in 13), and it may apply also to the assay of other transcription factors such as TFIIB and TFIIE (16).

Performance-testing of recombinant TFIID (TATA-binding protein) and SP1

The results described below illustrate how two of these three methods are used to test the Promega transcription factors TFIID (TATA-binding protein) and SP1 for their activation properties.

For the TFIID (TATA-binding protein) test, heat-treated HeLa nuclear extract is complemented with cloned, bacterially expressed (17) human TATA-binding protein (Promega Cat.# E3081) to rescue transcription at two different promoters: the adenovirus major late (AdML) promoter and the cytomegalovirus (CMV) promoter.

To test the recombinant SP1 protein, *Drosophila* embryo nuclear extracts are used to provide a transcriptional background with no endogenous homologue (18).

SP1 activation in *Drosophila* embryo nuclear extract

The primer extension analysis (shown in [Figure 1](#)) demonstrates the activation of an SP1-containing promoter above basal transcription levels with the addition of one footprint unit of human recombinant SP1 (Promega Cat.# E3391), containing 15-20ng of affinity-purified protein. The promoter contains three SP1 sites upstream from a viral TATA consensus sequence, and is readily transcribed by the *Drosophila* embryo nuclear extract in the absence of any added protein (lanes 1 and 2).

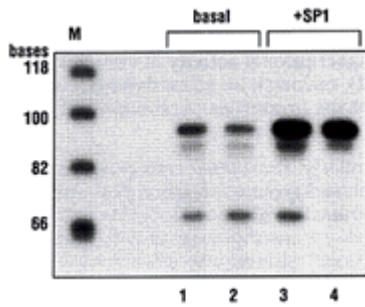


Figure 1. Activation of *in vitro* transcription by SP1 in *Drosophila* nuclear extract. In a standard transcription reaction, 1 μ l of *Drosophila* embryo nuclear extract was incubated with 0.3 μ g of a DNA template which contains 3 synthetic SP1 sites and a basal TATA box upstream from the CAT gene. The transcription products were extended with a radiolabeled primer complementary to the CAT transcript, producing a predominant primer extension product of approximately 90 bases. Extension products were resolved on an 8% denaturing polyacrylamide gel. The *in vitro* transcription reaction was performed in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 1 footprint unit of SP1. Lane M contains radiolabeled phiX174/*Hinf* I DNA size markers.

Primer extension reactions were performed on the mRNA obtained from duplicate transcription assays without added SP1 (lanes 1 and 2) and with SP1 (lanes 3 and 4). The level of activation is approximately 3-fold in these duplicates, as determined by liquid scintillation counting of the extension products excised from the gel. Titration experiments with SP1 added to the extract (R. Joshi, data not shown) demonstrate that the addition of 1 footprint unit of this factor gives optimal activation in our transcription reactions. (See [Figure 1](#) legend and Methods section, below.) Similar results with a fractionated *Drosophila* extract have been obtained with the same promoter by Dynlacht *et al.* (15).

TFIID (TATA-binding protein) activation in heat-treated HeLa nuclear extracts

Two different templates were assayed by run-off *in vitro* transcription in HeLa nuclear extracts, with and without heat-treatment ([Figure 2](#)).

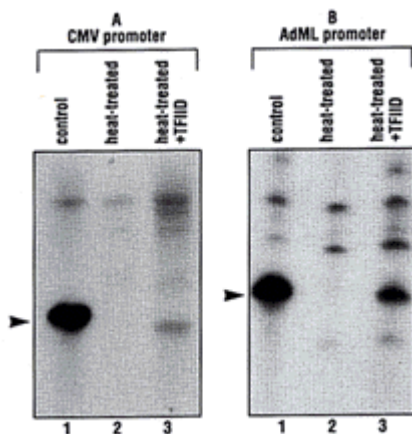


Figure 2. Effect of TFIID addition to heat-treated HeLa nuclear extract on *in vitro*

transcriptional activity of the CMV and AdML promoters. TFIID inactivation was achieved by heating HeLa nuclear extract at 47°C for 15 minutes. **Panel A:** A restriction fragment containing the CMV promoter was transcribed *in vitro* and the radiolabeled transcripts were resolved on a 6% denaturing polyacrylamide gel. Specific initiation of the CMV promoter produces the 371 nucleotide run-off product indicated by the arrow. Lane 1, control extract (not heated); lane 2, heat-treated extract; lane 3, heat-treated extract plus 1 footprint unit (15ng) of human recombinant TFIID (TATA-binding protein). **Panel B:** A linearized plasmid containing the AdML promoter (100ng) was transcribed *in vitro* and the run-off transcripts were analyzed on a 5% denaturing polyacrylamide gel. Specific initiation at the AdML promoter produces the 558 nucleotide run-off product indicated by the arrow. Lanes 1-3 are as described for panel A. The experiments shown in panel B were performed by Christopher Bartley and Peggy Farnham, University of Wisconsin-Madison, using an independently prepared HeLa nuclear extract.

In [Figure 2A](#), lane 1 shows the specific run-off transcript (see arrow) generated by the CMV template in HeLa nuclear extract under standard conditions. After heat treatment, all transcription is abolished from this template (lane 2). Approximately 25% of the CMV promoter activity is restored by the addition of 1 footprint unit (15-20ng) of human recombinant TFIID (lane 3).

The transcriptional activity of the AdML promoter is somewhat different; only the *specific* run-off transcripts (see arrow) are abolished by the heat treatment of the HeLa extract ([Figure 2B](#), lane 2). Interestingly, the addition of recombinant TFIID (one footprint unit) restores 50-75% of the AdML promoter activity, 2-3 times more than with the CMV promoter ([Figure 2B](#), lane 3; and additional data not shown).

Why is the rescue by TFIID quantitatively different on two different promoters?

The CMV promoter regains about one-half of the transcriptional activity achieved by the AdML promoter with the addition of recombinant TFIID to heat-inactivated HeLa nuclear extracts. The reason for this difference may lie in the relative heat lability of the upstream activator proteins which drive transcription from each promoter (19).

The heat-inactivation protocol (14) takes advantage of the special lability of endogenous HeLa TFIID. Other factors needed for the efficient expression of a given promoter also may be irreversibly inactivated by heating at 47°C (19). Thus, the rescue of transcriptional activity by recombinant TFIID addition may, in turn, reflect only the basal level of *in vitro* transcription.

In the case of the AdML promoter, efficient *in vitro* transcription is driven to a large extent by the USF protein (the adenovirus upstream stimulatory factor), which binds upstream of the AdML promoter TATA box between promoter positions -61 and -50 (19). USF, however, is known to be heat *stable*, with over 80% of its activity recoverable after a 10 minute incubation at 100°C (20).

Equivalence of footprint units and transcriptional activation units

Both activation experiments reveal an important equivalency between footprint units and optimal transcriptional activation. For both SP1 and TFIID activation, 1 footprint unit gave optimal

transcriptional enhancement or rescue results.

The titration of TFIID (TATA-binding protein) footprinting activity in [Figure 3A](#) illustrates how we determine the footprint unit designation. Promega's footprint unit is defined as the amount of recombinant protein required to give a complete protection pattern over the appropriate DNA binding site on the SV40 early promoter fragment. In the case of the recombinant TFIID, we have observed repeatedly that this protein does not give 100% protection from DNase I digestion (data not shown), unlike the recombinant SP1 protein ([Figure 3B](#)). Therefore, Promega defines full TFIID (TATA-binding protein) footprinting activity as approximately 80% or greater protection.

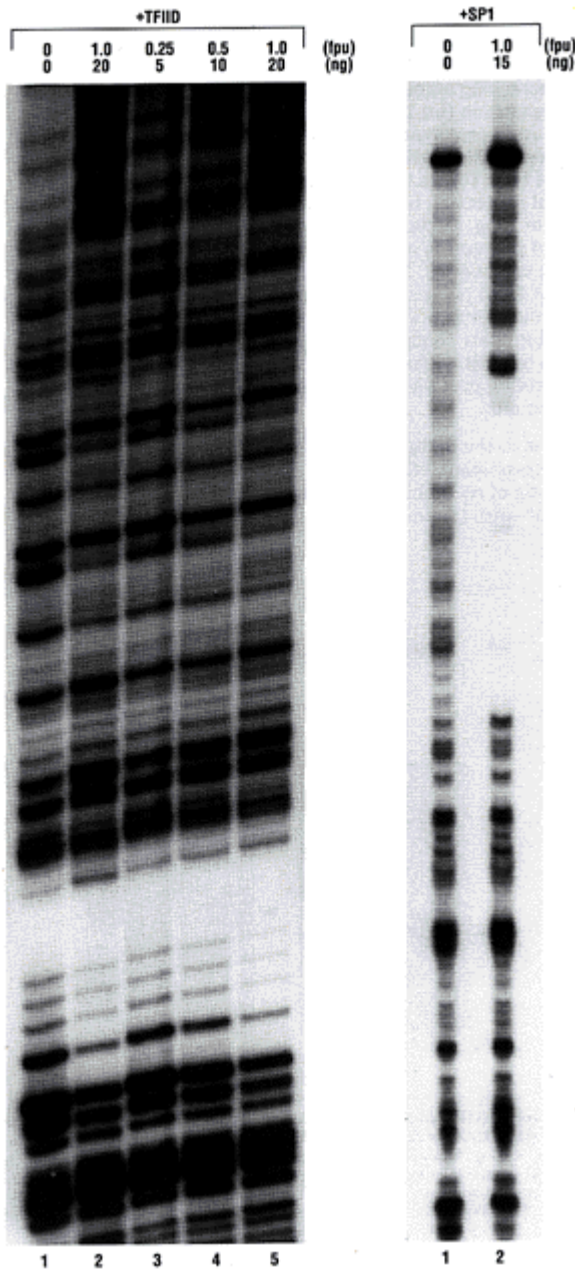


Figure 3. Footprints of TFIID (TATA-binding protein) and SP1 on an SV40 early promoter DNA template. Singly end-labeled 300bp SV40 promoter fragment (35 fmoles) was incubated in the presence or absence of TFIID (panel A) or SP1 (panel B) and partially

digested with DNase I. Fragments were resolved on a 6% polyacrylamide sequencing gel and detected by autoradiography. **Panel A:** Lane 1, no added protein; lane 2, 20ng TFIID (lot 080202); lanes 3-5, TFIID (lot 150301): 5ng, 10ng and 20ng, respectively. **Panel B:** Lane 1, no added protein; lane 2, 15ng SP1.

This level of protection is shown in [Figure 3A](#). Lane 1 shows the no-protein control and lane 2 shows the protection achieved by 1 footprint unit of Promega TFIID (TATA-binding protein, lot 080202). Lanes 3, 4, and 5 reveal the effect of adding increasing amounts of a different TFIID preparation (lot 150301), to the reaction. At approximately 5ng of added protein, very little of the TATA box is protected from DNase I digestion. No additional protection is visible with the addition of 10ng. At 20ng, however, significant protection over the TATA sequence is achieved. The addition of 30 or 40ng of TFIID protein increases the non-specific interaction of this protein with the template (data not shown). Based on this data, 20ng (1 μ l) of TFIID (TATA-binding protein, lot 150301) yields 1 footprint unit.

In contrast to the difficulties presented by the recombinant TFIID protein, the footprinting of recombinant SP1 is straightforward. ([Figure 3B](#), lane 2.)

Quality control: future specifications

As Promega expands its offerings of purified, recombinant transcription factors, we will attempt to define both the DNA binding and activation properties if both activities can be measured in a reliable, quantitative assay.

We are striving to support these products with the type of information our customers find most useful. Your feedback on our product support of transcription factors and extracts is very welcome. It will help us provide you with the experimental data you need in pursuing your research goals.

Methods

Transcription factors and nuclear extracts. The SP1 and TFIID (TATA-binding protein) used in experiments were manufactured at Promega. SP1 is expressed in HeLa by a recombinant vaccinia virus, and TFIID (TATA-binding protein) is expressed in bacteria. HeLa nuclear extracts were prepared according to a modification of the method of Dignam *et al.* (21). *Drosophila* embryo nuclear extracts were prepared according to the method of Wampler *et al.* (10).

***In vitro* transcription reactions.** For *Drosophila* embryo nuclear extract, 25 μ l *in vitro* transcription reactions were prepared in duplicate as described in Promega's Technical Bulletin 103. Each reaction contained 1 μ l of nuclear extract and 300ng of supercoiled DNA template. Following the *in vitro* transcription reaction, RNA products were subjected to primer extension analysis with a ³²P-labeled primer under standard conditions. The primer extension products were separated on a denaturing 8% polyacrylamide gel and analyzed by autoradiography.

For CMV transcription in HeLa nuclear extract, run-off transcripts were synthesized in 25 μ l reactions containing 3mM MgCl₂; 400mM ATP, CTP and UTP; 16 μ M GTP supplemented with ³²P- GTP; 58 μ g of HeLa nuclear extract protein; and 100ng of a 1.2kb restriction fragment containing the CMV promoter. The reactions were incubated at 30°C for 1 hour, 175 μ l stop solution (0.3M Tris-HCl, pH 7.4, 2mM EDTA, 0.3M sodium acetate, 0.5% SDS, 3 μ g/ml tRNA) was added, and the samples were extracted with phenol/chloroform and ethanol precipitated. One half of each sample was separated on a denaturing 6% polyacrylamide gel and analyzed by autoradiography. The specific run-off product was

cut out of the gel and quantitated by scintillation counting. For AdML transcription, reactions contained 6mM MgCl₂, 100µg of HeLa nuclear extract, and other components as described above for CMV transcription. The reactions were incubated at 25°C for 30 minutes and processed as described in the legend for Figure 2.

Footprinting assays. Footprinting was performed as described in Promega's certificates of analysis for SP1 and TFIID (TATA-binding protein) using a 300bp *Hind III/Kpn I* SV40 early promoter fragment as the template.

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Ordering Information:

Product	Size	Cat.#
HeLaScribe™ Nuclear Extract	40 reactions	E3091
<i>Drosophila</i> Embryo Nuclear Extract	100u	E3011
TFIID (human), TATA-Binding Protein	50fpu	E3081
AP1 (c-jun, human)	50fpu	E3061

AP2 (human)	50fpu	E3071
SP1 (human)	50fpu	E3391

1 fpu = 1 footprint unit (the amount of protein required to give full DNase I protection on a standard template).

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