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Sp1 activation of RNA polymerase II transcription complexes involves a heat-labile DNA-binding component

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We have identified a component of the eukaryotic RNA polymerase II transcriptional machinery that is more heat-labile than TFIID. DHFR transcriptional activity was severely reduced in 40°C heat-treated extracts in which TFIID was fully active. This heat-labile activity was required for the transcription of both TATA box and non-TATA box promoters that are activated by the transcription factor Sp1. Gel mobility shifts indicated that Sp1 DNA binding activity was heat-labile, and the addition of purified Sp1 to 40°C heat-treated extracts fully restored DHFR transcriptional activity. In contrast, the addition of Sp1 to 47°C heat-treated extract did not result in transcriptional activity from the DHFR promoter. We conclude that reduction in Sp1 DNA binding activity is partially responsible for the heat-sensitive loss of DHFR transcriptional activity, but that a second essential activity is also inactivated by 47°C heat-treatment. The discovery of this heat-labile component of Sp1 activation has two important implications in the analysis of transcriptional regulation. First, it demonstrates that heat-treated extracts are not appropriate for examination of the involvement of TFIID in the transcription of Sp1-activated promoters. Second, it explains the previously reported low-temperature optima for transcription from the DHFR promoter and demonstrates that transcriptional studies of Sp1-activated promoters should not be performed at 30°C.

Recent studies have indicated that RNA polymerase II promoters contain two elements important for positioning polymerase for accurate transcription initiation: the TATA box located 20–30 bp upstream of the start site, and the initiator element located at the start site (Sawadogo and Sentenac, 1990; Hariharan and Perry, 1990; Smale and Baltimore, 1989; Ayer and Dynan, 1988; Means and Farnham, 1990; Garfinkel et al., 1990). The relative importance of these elements varies in different promoters. The major late promoter of adenovirus 2 (MLP) is influenced by both elements, whereas the dihydrofolate reductase (DHFR) promoter lacks a TATA box but does contain an initiator element. Similar to many promoters that lack a TATA box, the DHFR promoter is GC-rich. Many GC-rich promoters contain multiple binding sites for the transcription factor Sp1, with one site usually located 40 to 90 bp upstream of the transcription initiation site (Kadonaga et al., 1986). Sp1 binds to multiple GGGCGG sequence elements and was identified as an activator of in vitro transcription of the SV40 early promoter (Dynan and Tjian, 1983b; Kadonaga et al., 1987). The DHFR promoter contains 10

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consensus Sp1 binding sites (McGrogan et al., 1985). Partially purified Sp1 has been shown to bind to the four GC boxes nearest to the initiation site and to activate DHFR transcription (Dynan et al. 1986). Through deletion analysis, we have shown that one Sp1 site located 60 bp upstream of the DHFR initiation site is sufficient for accurate transcription (Farnham and Means, 1990). Additionally, the combination of a single Sp1 site and the DHFR initiator element creates a functional promoter when inserted into a plasmid vector (Means and Farnham, 1990). The lack of an obvious TATA box and a kinetic analysis of DHFR transcription complex formation (Farnham and Schimke, 1986; L. J. Schilling, unpublished data) suggest that TFIID does not bind to the DHFR promoter region; however, it remains possible that TFIID can be involved in transcription of the DHFR gene via protein–protein interactions. Using an assay that relies on the heat-inactivation of TFIID, we asked if TFIID can stimulate transcription from non-TATA box promoters. We found that cloned TFIID was unable to stimulate activity from several non-TATA box promoters in the 47°C heat-treated extract. These studies led to the discovery that optimal transcriptional activity from some promoters required an activity that was more heat-labile than TFIID. Our results indicate that the addition of TFIID to 47°C heat-treated extracts cannot reactivate Sp1-activated promoters that lack a TATA box due to the inability of heat-treated Sp1 to bind DNA.

Materials and methods

In vitro transcriptions

All reactions contained 100 μg of HeLa nuclear protein, 10 nM DNA, 6 mM $MgCl_2$ (except for MDR1 transcriptions which contained 2 mM $MgCl_2$), 20 mM Hepes (pH 7.9), and previously described buffers and nucleotide concentrations (Dignam et al., 1983; Farnham and Kollmar, 1990). Reactions were performed at 24–25°C for 30 minutes. Radiolabeled RNA made in vitro using SP6 RNA polymerase was included in the stop buffer as an internal control to correct for recovery of RNA. Signals were quantitated by scanning the autoradiographs with an LKB soft laser densitometer and integrating the peaks with a Numonics Corp. 1224 electronic digitizer. One hundred μl aliquots of extract were heat-treated in a waterbath at 30, 35, 40, or 47°C for 10 minutes, quickly frozen in liquid nitrogen, and stored at −70°C.

Plasmid constructs

Promoter coordinates, restriction enzyme fragments, and run-off transcript size for the different mammalian promoters used in the in vitro transcription assays are given in Table 1. All promoter constructs have been described previously (see Table 1) except for pATS528 and pCMV. The construct pATS528 contains sequences extending from −259 to +270 of the MLP of adenovirus 2 and was created by insertion of a TaqI/Sau3A fragment from pLax (Dynan and Tjian, 1983) into the AccI and BamHI sites of pBSM13+ (Stratagene). The construct pCMV contains sequences from −806 to +15 of the CMV promoter inserted into the EcoRI and BamHI sites of pUC9. The synthetic promoters containing the 21 bp repeats of SV40 plus an oligonucleotide for the MLP TATA box (Sp1-TATA), the 21 bp repeats plus the terminal deoxynucleotidyltransferase (TdT) initiator element (Sp1-Inr), or the TATA oligonucleotide and the TdT Inr (TATA-Inr) have been described previously (Smale et al., 1990). The construct pGC contains a single Sp1 consensus binding site consisting of the synthetic oligonucleotide 5′ GATCGGGGCGGGGC 3′ and its complement inserted into the BamHI site of pUC19. The NdeI/HindIII fragment from pGC was purified by electroelution after polyacrylamide gel electrophoresis for use in gel mobility shift assays. A 38 bp synthetic oligonucleotide consisting of sequences from the SV40 promoter spanning +52 to +84 (containing three Sp1 binding sites) was also used in gel mobility shift assays.

Gel mobility shift assays

The binding of Sp1 to DNA was performed as previously described (Letovsky and Dynan, 1989) with the following modifications. Two μg sonicated salmon sperm DNA and 100 ng of concatemerized binding sites for either Sp1 or TFIID were incubated with 12 μl of binding buffer (7.1 mM Hepes [pH 8.0], 3.6 mM $MgCl_2$, 100 mM KCl, 5.7% glycerol, and 0.03% NP40) containing 6 μg nuclear extract for 10 minutes at 21°C. Five μl (approximately 0.25 ng) of radiolabeled DNA (corresponding to either the NdeI/HindIII fragment from pGC or the 38 bp double-stranded oligonucleotide con-

Table 1. Description of promoters.

Genomic sequences (+1 is the transcription initiation site) present on each template, the size of the transcript, the presence of a TATA box, and the number of consensus Sp1 binding sites are indicated. Templates were prepared by digestion of the plasmid DNA with the indicated restriction enzymes.

*Promoter**	*Coordinates*	*Transcript*	*TATA*	*Sp1*	*Plasmid*	*Enzymes*	*Reference*
DHFR (wt)	−356/+66	260	No	4	pST410	PvuII	Farnham and Means, 1990
DHFR (1 repeat)	−87/+52	290	No	1	pBSpro19	PvuII	Farnham and Means, 1990
MDR1	−439/+280	285	No	0	pP3	PstI/BglII	Cornwell, 1990
REP	−190/+270	270	No	6	pRT10−	EcoRI/SmaI	Schilling and Farnham, 1989
CAD	−332/+406	436	No	2	pC76B	BamHI/HindIII	Farnham and Kollmar, 1990
SV40 early	−137/+408	408	Yes	6	pSVS	NdeI/SphI	Fromm and Berg, 1982
MLP	−259/+270	558	Yes	0	pATS528	HaeII	†
H2b	−175/+520	520	Yes	0	pH2b	HindIII/BamHI	Steinberg et al., 1990
IRF	−299/+225	225	No	2	pIRF-CAT	PstI	Miyamoto et al., 1988
CMV	−806/+15	371	Yes	2	pCMV	EcoRI/BamHI	†

* The promoters used in this study are as follows: DHFR (from the mouse dihydrofolate reductase gene); MDR1 (from the human multidrug resistance gene 1); REP (the upstream opposite strand promoter from the mouse DHFR locus that drives the REP1 gene); CAD (from the syrian hamster carbamoylphosphate synthetase-aspartate transcarbamylase-dihydroorotase gene); SV40 early (the sequences driving the simian virus 40 early transcripts); MLP (the sequences driving the major late transcripts of adenovirus 2); H2b (from the histone H2b gene); IRF (from the interferon regulatory factor gene 1); CMV (the sequences driving the major immediate early transcripts from cytomegalovirus).

† See Materials and Methods for description of promoter construct.

taining three Sp1 binding sites from the SV40 genome) was then added and the incubation continued for 10 minutes. The reactions were electrophoresed for 90 minutes on a 4% polyacrylamide gel which had been pre-electrophoresed for 60 minutes. The gel buffer was 0.25X TBE (Maniatis et al., 1982). The NdeI/HindIII fragment and the 38 bp oligonucleotide were phosphorylated with T4 polynucleotide kinase and [γ-^{32}P]ATP, as previously described (Maniatis et al., 1982).

Results

Cloned TFIID cannot activate transcription from non-TATA box promoters

TFIID in HeLa nuclear extract can be inactivated by a 10-minute incubation at 47°C without inactivating other general transcription factors or RNA polymerase II (Nakajima et al., 1988). Analysis of two nonTATA box promoters, dihydrofolate reductase (DHFR) and multidrug resistance-1 (MDR1), demonstrated that their transcription was also reduced in 47°C heat-treated extract (Fig. 1). This suggested that the heat-treatment inactivated a factor(s) required for DHFR and MDR1 transcription. To determine if the inactivated factor(s) was TFIID, an amino-octyl-agarose 0.2 M KCl fraction from a HeLa TFIID purification (Nakajima et al., 1988) and cloned human TFIID (Hoffmann et al., 1990) were added to the heat-treated extract. Both sources of TFIID restored activity for the MLP, while transcription from the MDR1 promoter was stimulated by the amino-octyl-agarose fraction only. Transcription from the DHFR promoter was not stimulated by either source of TFIID. Similar results were obtained for the non-TATA box CAD promoter (P. J. Farnham, unpublished results). The differential activation of MDR1 and DHFR by the amino-octyl-agarose column fraction suggested that an additional heat-sensitive activity that could not be substituted for by TFIID was required for DHFR transcription.

An activity required for DHFR transcription is more heat-labile than TFIID

To determine if DHFR transcription had the same sensitivity to heat-treatment as TFIID inactivation, the transcriptional activity of the MLP and the DHFR promoter was examined in nuclear extracts that had been heat-treated for 10 minutes at 30, 35, 40, or 47°C (Fig. 2). The MLP was fully active in the 40°C heat-treated extract, but was inactive in the 47°C heat-treated extract. In contrast, transcription from the DHFR promoter was reduced in the 30, 35, and 40°C heat-treated extracts. This increased sensitivity to heat-treatment suggested that the DHFR promoter required an activity that was more heat-labile than TFIID. Thus, it was not possible for TFIID to reactivate transcription of DHFR due to the additional requirement for the heat-labile activity.

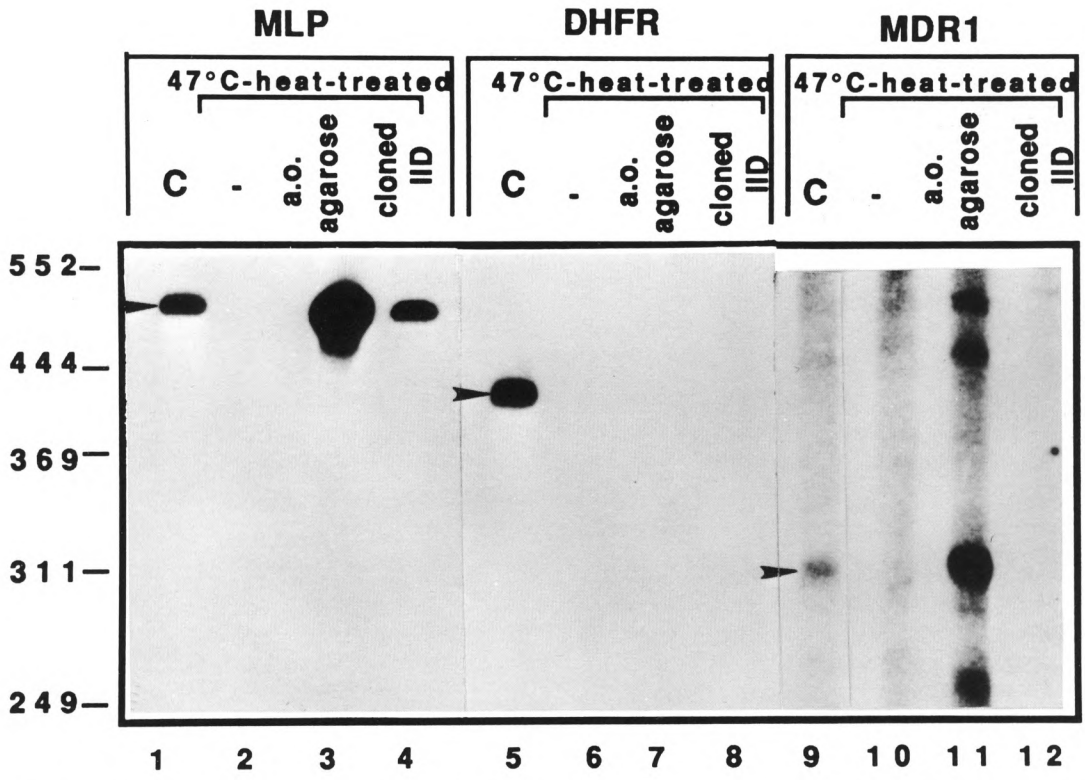

Figure 1. Cloned TFIID cannot reactivate correctly initiated transcription from non-TATA box promoters in heat-treated nuclear extracts. Transcriptions were performed on the MLP (lanes 1–4), the DHFR (lanes 5–8), and the MDR1 (lanes 9–12) promoters. The bands corresponding to correctly initiated transcription are marked with an arrowhead. The type of HeLa nuclear extract and the source of TFIID is indicated above the lanes.

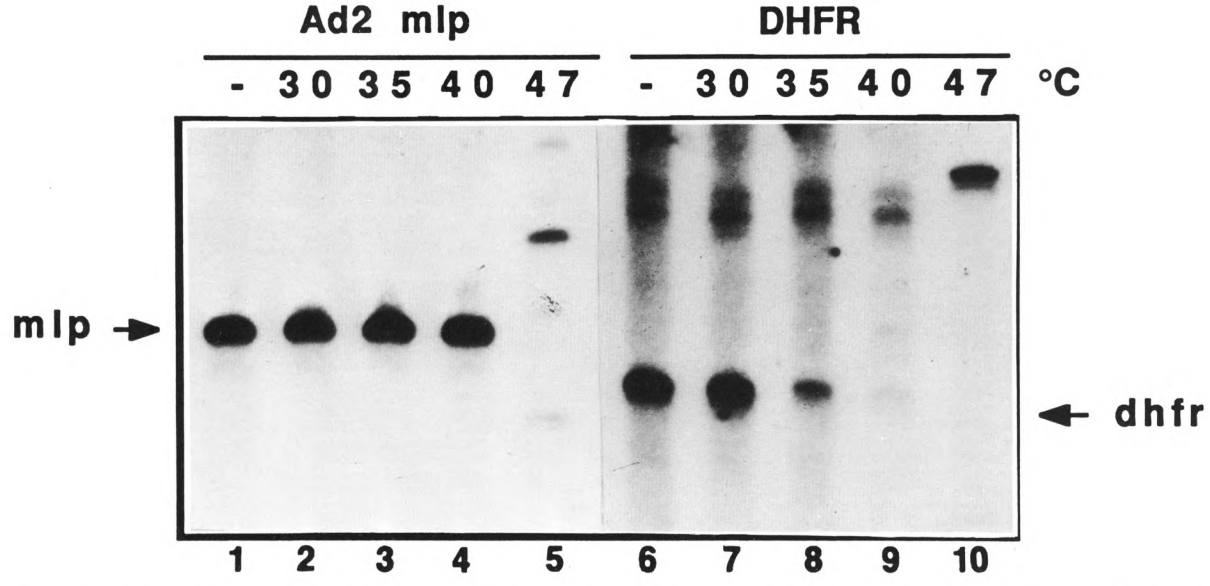

Figure 2. A heat-labile activity is required for DHFR transcription. The run-off transcripts from the MLP (lanes 1–5) and the DHFR promoter (lanes 6–10) are shown in the control and heat-treated nuclear extracts.

Many promoters utilize the heat-labile activity

To determine if the heat-labile activity was a general factor for the class of non-TATA box promoters, we examined a collection of promoters that either contained or lacked a consensus TATA box (Table 1). Each promoter was first transcribed in unheated control extract to ensure that transcripts corresponding to the correct initiation site were obtained (Fig. 3A). Analysis of the promoters in heat-treated extracts demonstrated that they fell into two distinct classes (Fig. 3B). The activity from the MLP, H2b, and MDR1 promoters was not reduced by 40°C heat-treatment of the nuclear extract. However, the activity from the DHFR, CAD, SV40 early, CMV, REP, and IRF promoters was greatly reduced in the 40°C heat-treated extract. The ability of heat-treated extracts to transcribe the different promoters did not correlate with the overall activity of the promoters, nor did it correlate with the presence or absence of a consensus TATA box.

The existence of a heat-labile activity explains our previous finding that DHFR transcription is maximal at 24°C (Farnham and Schimke, 1986), a temperature much lower than 30°C which is normally used for in vitro transcription assays. Although the temperature optima for transcription from all the promoters examined have not been determined, a low temperature optimum for transcription has also been observed for the CAD promoter (Farnham and Kollmar, 1990), for the REP promoter (L. J. Schilling, unpublished data), and for the CMV promoter (P. J. Farnham, unpublished data). It is probable that the low temperature requirement for optimal transcription is at least partially due to a heat-labile activity used by this class of promoters, but not by the MLP. Transcription from the MDR1 promoter does not require the heat-labile activity and has a temperature optimum between 28°C and 37°C (Cornwell, 1990). This temperature optimum is higher than the optima for DHFR and CAD, but close to the 30°C temperature optimum reported for the MLP (Dignam et al., 1983).

It is important to note that the involvement of this heat-labile activity in the transcription of a promoter may not be apparent if transcriptions are performed at 30°C, due to the inactivation of the heat-labile activity during the preincubation of template and extract. Transcription from the CMV promoter at both 24°C and 30°C was examined in the different heat-treated extracts (Fig. 3C). A 15-minute preincubation of template DNA and extract was allowed for complex formation before the addition of nucleotides, followed by a 15-minute reaction time. Although the sensitivity of CMV transcription to the heat-treatment was obvious at transcriptions performed at 24°C, very little difference was detected when transcriptions were performed at 30°C. In fact, the level of CMV promoter activity in transcription reactions performed at 30°C using the unheated extract was approximately the same as the level in reactions performed at 24°C using extract that had been pre-heated at 40°C for 10 minutes. It should be noted that preincubation at 30°C of template DNA and extract for longer than 15 minutes results in even greater inactivation of the heat-labile factor, resulting in correspondingly less transcriptional activity (P. J. Farnham, unpublished data).

The presence of Sp1 binding sites correlates with heat sensitivity

If a specific DNA sequence element correlated with the heat sensitivity, then deletion of this element from the promoter region should reduce the heat sensitivity of that construct. We have previously mapped the DNA regulatory elements of the DHFR promoter (Farnham and Means, 1990) and could thus test specific constructs that lack individual protein binding sites. The promoter construct that was used in the initial heat sensitivity assays contains four 48 bp repeats (each of which contain a binding site for Sp1), a regulatory element that spans −11 to +10 (a binding site for HIP1), and a protein binding site that spans +46 to +56 (Fig. 4A). Promoter constructs specifically mutated at each of these sites were then tested in the heat-treated extracts (Fig. 4B). Mutation of the HIP1 site reduced overall DHFR transcriptional activity but did not result in decreased heat sensitivity. Reactivation of 35°C heat-treated nuclear extract confirmed that HIP1 is not the heat-labile factor since a flowthrough fraction from a DHFR initiator element-DNA oligonucleotide column contained the heat-labile activity, but the fractions having HIP footprinting activity did not (P. J. Farnham, unpublished data). Deletion of the DSE (the protein binding site in the 5′ un-

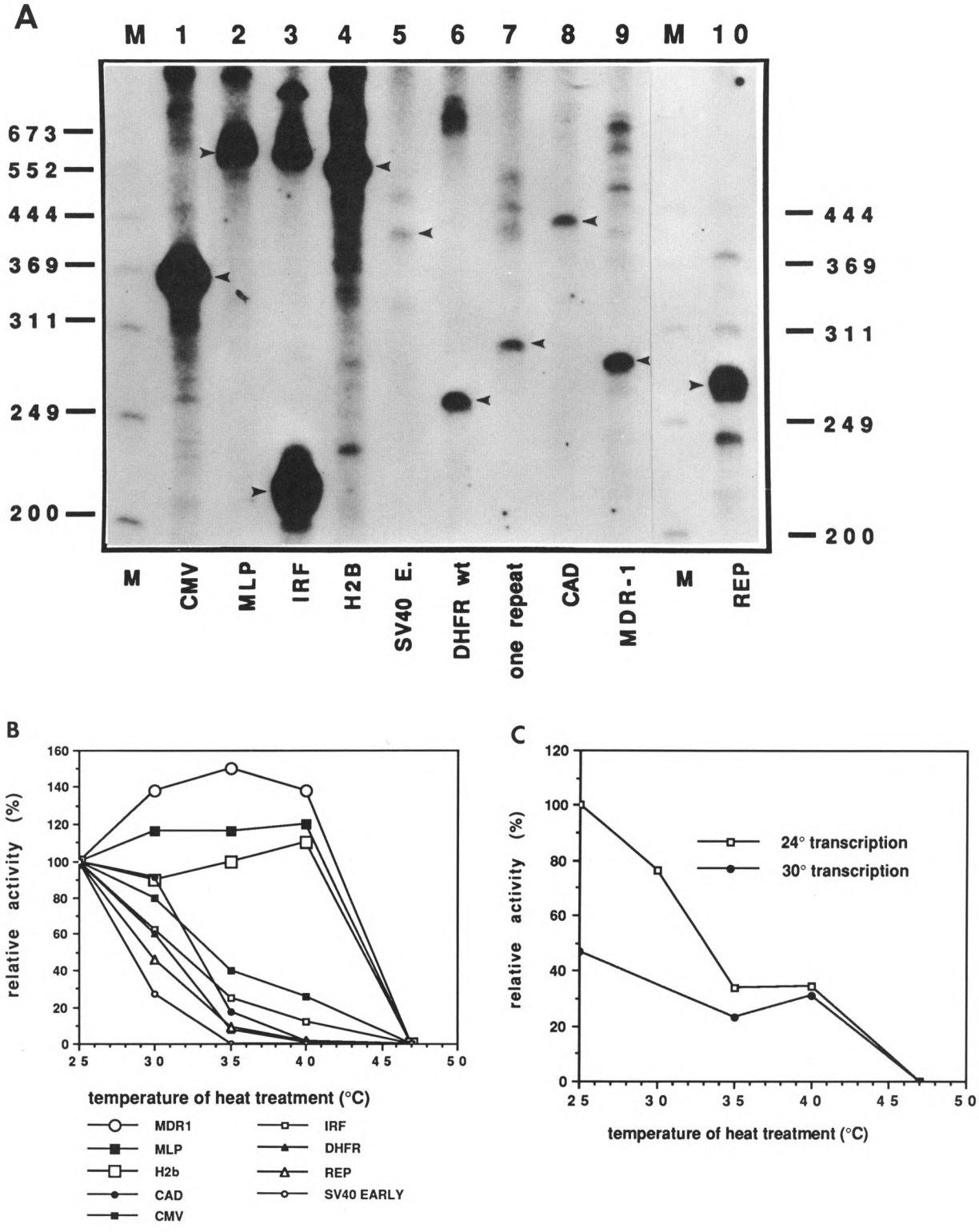

Figure 3. Transcriptional activity of different promoters in the heat-treated extracts. **A.** The band corresponding to the correctly initiated transcript from each promoter in control extract is indicated by the arrowhead in each lane. The transcriptional activity of all promoters can be directly compared, except for the activity of the REP template which was analyzed on a separate gel. Size markers in bp are at the left for lanes 1–9 and at the right for lane 10. **B.** Transcriptional responses of all the promoters in the heat-treated extracts. **C.** Transcriptional response in the heat-treated extracts of the CMV promoter in reactions performed at 24°C or 30°C. The activity is graphed relative to the activity of the CMV promoter in reactions performed at 24°C in the control extract. In these reactions, template DNA and extract were preincubated for 15 minutes to allow complex formation prior to the addition of nucleotides.

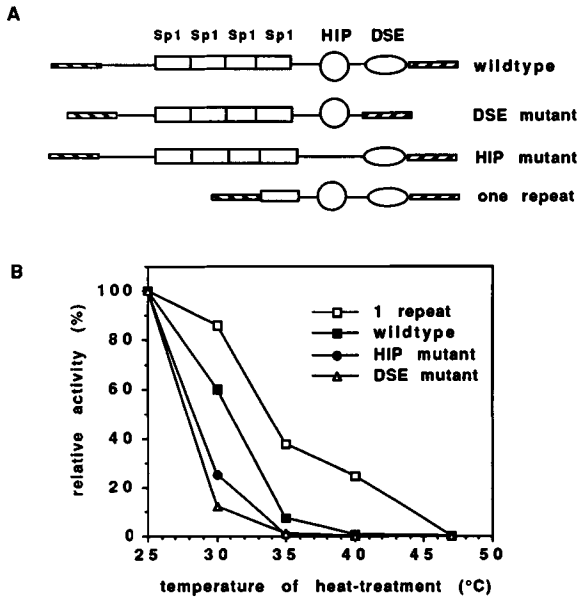

Figure 4. Analysis of mutant DHFR promoters. The transcriptional responses of different DHFR promoter mutants in the heat-treated extracts is shown. Transcriptional activity in the different extracts is graphed relative to the activity of the same construct in the control extract. The overall activity of the HIP mutant and the DSE mutant in control extract is approximately 10–20% of the wildtype signal in control extract. The activity of the one repeat mutant in control extract is 50% of the activity of the wildtype construct in control extract.

translated region) again reduced transcriptional activity of the DHFR promoter, but also did not decrease the heat sensitivity of the DHFR promoter. However, deletion of three of the four Sp1 sites reduced the heat sensitivity of the promoter, suggesting that Sp1 was involved in the heat sensitivity. Deletion of all four Sp1 sites results in a non-functional DHFR promoter (Farnham and Means, 1990); therefore we could not determine the heat sensitivity of a DHFR promoter construct that lacks all Sp1 sites. However, the fact that deletion of three Sp1 sites reduced the heat sensitivity, whereas deletion of the other binding sites increased the heat sensitivity of the DHFR promoter, suggested that Sp1 sites were involved.

Inspection of the other promoters examined revealed that although reduced transcription in the 40°C heat-treated extract did not correlate with the presence or absence of a TATA box, it did correlate with the presence of an Sp1 consensus binding site (Table 1). In general, promoters that have many Sp1 sites (such as SV40 early and REP) are more heat-sensitive than promoters that have only a few Sp1 sites (such as the one repeat DHFR construct and CMV). However, because each of the promoters tested is very different, we could not directly determine if the same DNA binding protein was the heat-labile activity. We therefore tested a series of synthetic promoters either containing or lacking Sp1 sites (Fig. 5). First, synthetic promoters consisting of Sp1 sites activating two different initiator elements were tested. The construct denoted Sp1-TATA consists of a restriction fragment containing the 21 bp repeats of SV40 inserted upstream of a consensus TATA box, whereas the construct denoted Sp1-Inr contains the same region from SV40 inserted upstream of the TdT initiator element (Smale et al., 1990). Both Sp1-TATA and Sp1-Inr were heat-sensitive, again demonstrating that the heat-labile activity is required for both TATA and non-TATA box promoters. However a construct containing a consensus TATA box upstream of the TdT Inr denoted TATA-Inr (Smale et al., 1990) did not require the heat-labile activity. Similar to MLP, MDR1, and H2B, transcription from TATA-Inr increased after heat-treatment of the extract.

The ability of Sp1 to bind DNA is reduced in the heat-treated extracts

The correlation between Sp1 sites and heat sensitivity suggested that some component of Sp1

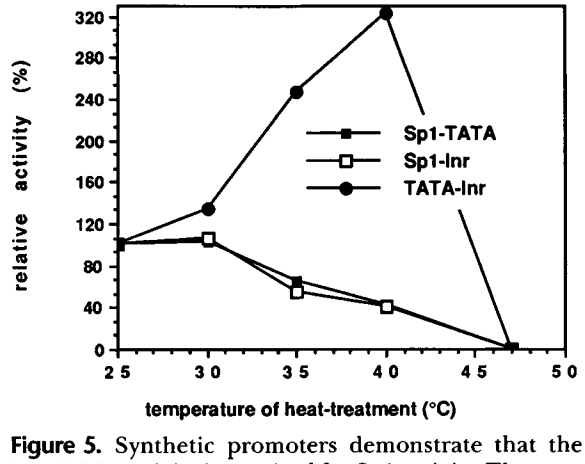

Figure 5. Synthetic promoters demonstrate that the heat-labile activity is required for Sp1 activity. The transcriptional responses in the heat-treated extracts of synthetic promoters containing Sp1 sites activating transcription from a TATA box (Sp1-TATA) or an initiator element (Sp1-Inr) are compared to the response of a synthetic promoter that lacks Sp1 sites (TATA-Inr).

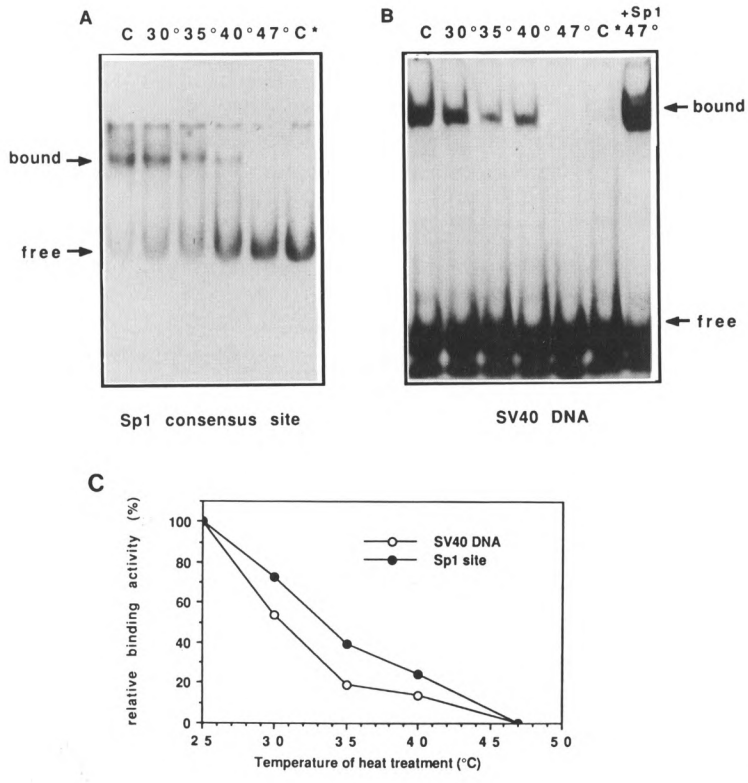

Figure 6. Binding of Sp1 to DNA is heat-sensitive. A 278 bp NdeI/HindIII fragment containing a single Sp1 consensus site (**A**) or a 38 bp synthetic oligonucleotide containing 3 Sp1 sites from the SV40 promoter (**B**) were used in gel mobility shift assays with control extract and extracts that had been heat-treated for 10 minutes at 30, 35, 40, or 47°C. All reactions contained 100 ng of concatemerized oligonucleotides of the sequence AATTCTATAAAAGCG except for lanes C* which contained 100 ng of concatemerized Sp1 binding site oligonucleotides of the sequence GATCGGGGCGGGGC. Five ng of purified Sp1 were added to the 47°C heat-treated extract in the last lane of **B**. The results from two separate gel mobility shift experiments for each probe were averaged, and the relative binding activity (compared to the activity in the control extract) was graphed in **C**.

activation might be perturbed by the heat-treatment. Therefore, we examined the ability of Sp1 to bind to DNA in the heat-treated extracts. A 278 bp DNA fragment containing a single Sp1 consensus site (Fig. 6A) and a 38 bp SV40 DNA sequence oligonucleotide containing three Sp1 binding sites (Fig. 6B) were used in gel mobility shift assays. The DNA fragments migrated with a slower mobility after incubation with control HeLa nuclear extract, demonstrating that a protein bound to each fragment. This binding activity was shown to be due to Sp1 since it could be competed by an oligonucleotide consisting of concatemerized Sp1 binding sites (lanes C*), but not by an oligonucleotide consisting of concatemerized TFIID binding sites (lanes C). The ability of Sp1 to bind to these DNA fragments was severely reduced in the heat-treated extracts (Fig. 6C), suggesting that loss of Sp1 DNA binding activity was responsible for the reduction in transcriptional activity of the heat-sensitive promoters. To determine if the heat-treatment had activated an inhibitor of Sp1 binding, purified Sp1 was added to the 47°C heat-treated extract (Fig. 6B, lane 47° +Sp1). Since Sp1 could bind to the DNA when added after heat-treatment, this suggested that Sp1 itself was inactivated and that the loss in activity in the heat-treated extracts was not due to an inhibitor of Sp1 binding.

Reactivation of transcriptional activity in heat-treated extracts by Sp1

Since purified Sp1 could bind to DNA when added to a heat-treated extract, this led us to test whether the addition of Sp1 could restore transcriptional activity from the DHFR promoter in the heat-treated extracts. Control, 40°C, and 47°C heat-treated extracts were examined for activity from the DHFR promoter before and after the addition of 10 ng of purified Sp1 (Fig. 7A). The addition of Sp1 could restore full DHFR transcriptional activity to the 40°C heat-treated extract, demonstrating that the inactivation of Sp1 was responsible for the loss of activity from the DHFR promoter. In contrast, the addition of Sp1 did not result in DHFR promoter activity in the 47°C heat-treated extract, suggesting that another essential component of the transcriptional machinery was inactivated in this extract. We have not yet been successful in restoring DHFR transcriptional activity to

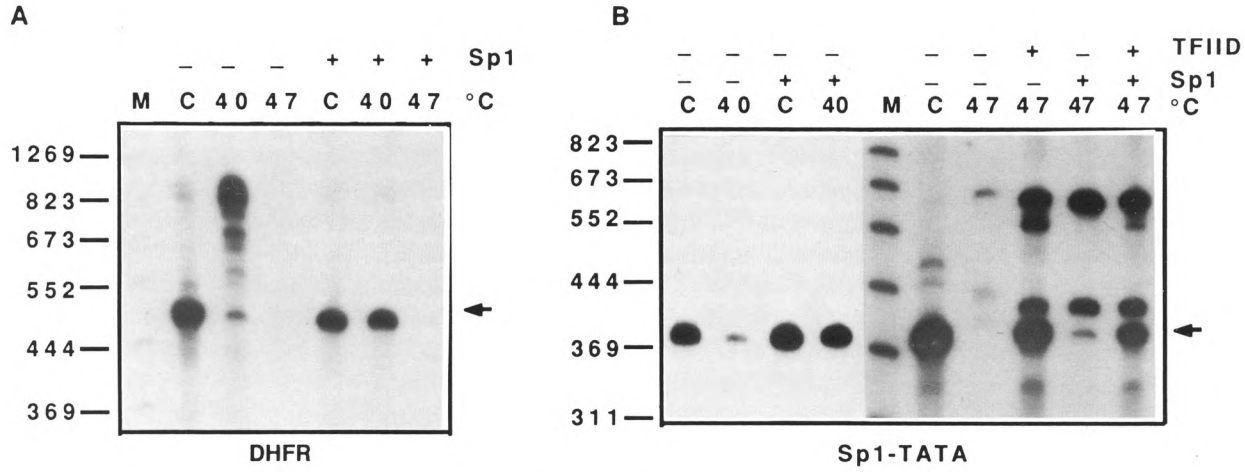

Figure 7. Addition of purified Sp1 to heat-treated extracts. **A.** The transcriptional response of the DHFR promoter was examined in control extracts and extracts heated for 10 minutes at 40 or 47°C. The reactions were supplemented with purified Sp1 as indicated. The correctly initiated transcript is indicated by the arrow. **B.** The transcriptional response of the Sp1-TATA promoter was examined in control extracts and extracts heated for 10 minutes at 40 or 47°C. The reactions were supplemented with purified human Sp1 or bacterially expressed yeast TFIID as indicated. The correctly initiated transcript is indicated by the arrow.

the 47°C heat-treated extract by the addition of TFIID plus Sp1 (data not shown), suggesting that as yet another factor, either instead of or in addition to TFIID, is required to restore DHFR transcriptional activity to 47°C heat-treated extracts.

We next tested whether the addition of Sp1 to the heat-treated extracts could restore transcription from the Sp1-TATA promoter (Fig. 7B). First, control and 40°C heat-treated extracts were examined for activity from the Sp1-TATA promoter before and after the addition of purified Sp1. The addition of Sp1 could restore full Sp1-TATA promoter activity from the 40°C heat-treated extract. Others have shown that the addition of yeast TFIID can restore activity to 47°C heat-treated extracts for Sp1-activated promoters such as E1B (Schmidt et al., 1989) and Sp1-TATA (Smale et al., 1990). We have reproduced the results for Sp1-TATA (Fig. 7B) which again demonstrate that the addition of yeast TFIID alone can restore Sp1-TATA transcription in the 47°C heat-treated extract. The addition of both Sp1 and TFIID to the 47°C heat-treated extract also restored high levels of activity; however, transcription was not increased above the signal detected by the addition of TFIID alone. Finally, the addition of Sp1 to the 47°C heat-treated extract resulted in a small amount of correctly initiated transcription from Sp1-TATA, suggesting that accurate initiation could occur, albeit at a low level, without the addition of TFIID.

Discussion

Our experiments provide evidence for the existence of a heat-labile activity of the eukaryotic RNA polymerase II transcriptional machinery required for transcription of several promoters that either contain (CMV, SV40 early) or lack (DHFR, CAD, IRF, REP) a TATA box. Importantly, all of these promoters contain consensus binding sites for the transcription factor Sp1, suggesting that some component of Sp1 activation is the heat-sensitive activity. Since purified Sp1 can restore activity to 40°C heat-treated extracts for both the DHFR and the Sp1-TATA promoters, this indicates that heat-treatment has not activated an inhibitor of Sp1 function. Instead, the activity of the Sp1 protein itself has been reduced. The transcriptional effects of Sp1 are mediated through the binding of Sp1 to DNA via a zinc finger motif (Kadonaga et al., 1987), and activation of transcription through glutamine-rich domains (Courey et al., 1988). Our data suggest that one of these functions has been impaired in heat-treated extracts.

We have shown that heat-treated Sp1 is de-

ficient in DNA binding activity. Gel mobility shift assays revealed that protein binding to Sp1 consensus sequences is not detectable after heating the extract to 47°C. DNAse I footprinting studies also indicated that protein binding to the Sp1 consensus sites in the DHFR promoter is eliminated after 47°C heat-treatment of HeLa nuclear extract (A. L. Means, unpublished data). This reduction of binding to the Sp1 sites in heat-treated extracts correlates well with the transcriptional profile of promoters that are activated by Sp1. The ability of Sp1 to bind DNA could be reduced by either differential protein modification in the heat-treated extracts or by a heat-induced conformational change in a DNA binding domain. Sp1 has been shown to be post-translationally modified by glycosylation and phosphorylation. However, neither the extent of glycosylation nor the degree of phosphorylation affects binding of Sp1 to DNA (Jackson and Tjian, 1988; Jackson et al., 1990). Sp1 is phosphorylated by a DNA-activated protein kinase (Jackson et al., 1990, Carter et al., 1990, Lees-Miller et al., 1990). This protein kinase is fairly stable to heat-treatment in the absence of DNA (greater than 25% activity remains after incubation for 10 minutes at 50°C), but it displays an increased rate of inactivation in the presence of DNA. In contrast, the heat-sensitivity of Sp1-activated promoters is stabilized by the addition of template DNA to the extract during the heat-treatment (R. Kollmar, unpublished data). Thus, the loss of DNA binding activity of the heat-inactivated Sp1 is probably not due to changes in the extent of glycosylation or phosphorylation of Sp1. Sp1 is present in HeLa cells in two forms that bind DNA with low versus high affinity (Kadonaga et al., 1988). The region of Sp1 that confers high affinity binding is distinct from the zinc finger region required for sequence-specific, low affinity binding. It is possible that heat-treatment has altered one or both of these DNA binding domains. Disruption of high affinity binding might result in a form of Sp1 that could no longer bind stably to the promoter construct under in vitro transcription conditions. Alternatively, conformational changes in the zinc finger region may result in loss of sequence-specific DNA binding ability.

We cannot directly assess the effects of heat-treatment on the Sp1 activation domains. However, promoters that contain Sp1 sites plus a TATA box are active in 47°C heat-treated extracts after addition of TFIID. For example, the E1B promoter, containing a single Sp1 site and a TATA box, and the synthetic Sp1-TATA promoter can be reactivated by the addition of yeast TFIID to 47°C heat-treated extracts (Schmidt et al., 1989; Smale et al., 1990). We have shown that addition of active Sp1 to the 47°C heat-treated extract along with yeast TFIID does not result in activity from the Sp1-TATA promoter above that seen by the addition of TFIID alone. There are at least two explanations for these results. One explanation for the transcriptional activity from Sp1-TATA in the 47°C heat-treated extract after addition of TFIID is that activity from this promoter may not depend upon activation by Sp1. Smale et al. (1990) have shown that a construct containing only a TATA box is active after addition of yeast TFIID to heat-treated extract. Thus, it is possible that heat-inactivation may have impaired Sp1 DNA binding and activation functions, and that the activity from the Sp1-TATA promoter in 47°C heat-treated extracts supplemented with TFIID is simply the result of basal transcription driven by the TATA box.

The second possibility is that, in contrast to the DNA binding function, the ability of Sp1 to activate transcription may not be significantly affected by the heat-treatment. If so, then heat-treated Sp1 may be able to activate transcription if it is recruited into a transcription complex via direct or indirect interactions with another DNA binding factor such as TFIID (Pugh and Tjian, 1990). A mechanism of Sp1 activation that is independent of DNA binding is not unprecedented, since a DNA binding-deficient mutant of Sp1 has been previously shown to interact with bound Sp1 to superactivate transcription (Courey et al., 1989). If Sp1 and TFIID interact (either directly or indirectly), then perhaps the addition of either Sp1 or TFIID to the 47°C heat-treated extract could lead to transcription from the Sp1-TATA promoter. Accordingly, we found that the addition of Sp1 to the 47°C heat-treated extract did restore a very small amount of accurate initiation from the Sp1-TATA promoter, suggesting that perhaps Sp1 was bringing a DNA-binding deficient TFIID into the transcription complex. The failure to restore full activity could be due to the heat-inactivation of a TFIID function other than DNA binding. The ability of Sp1 to recruit TFIID into a transcription complex could be tested directly in

reconstitution experiments using TFIID DNA binding mutants. Alternatively, the kinetics of Sp1 binding to and dissociation from the template may not be optimal for formation of a stable transcription complex in the absence of TFIID binding. In contrast to TFIID which dissociates from the DNA with a half-life of 1 hour at 25°C, Sp1 dissociates very rapidly with a half-life of 1 minute at 25°C (Schmidt et al., 1989). Thus, the binding of Sp1 alone may not be sufficient to create a stable transcription complex.

We have shown that accurate initiation from the DHFR promoter in 47°C heat-treated extract cannot be restored by TFIID, and others have published similar results using the Sp1-Inr promoter (Smale et al., 1990). Further, the addition of Sp1 plus yeast TFIID also does not restore accurate initiation from the DHFR promoter in 47°C heat-treated extract (data not shown), suggesting that at least one additional component has been inactivated by the heat-treatment that cannot be restored by the addition of Sp1 or TFIID. Thus, determining the involvement of TFIID in the transcriptional regulation of the DHFR gene must await further characterization of the components required to reactivate DHFR transcription.

In conclusion, the characterization of a heat-labile Sp1 DNA binding activity has two important implications in the analysis of transcriptional regulation. First, these experiments demonstrate that 47°C heat-treated extracts are not optimal for analysis of the involvement of TFIID in the transcription of promoters that are dependent on Sp1-activation. Heat-treatment at 47°C does not selectively inactivate TFIID, but also destroys the ability of Sp1 to bind to DNA. This inactivation of the DNA binding ability of Sp1 by heat-treatment is helpful in understanding the inconsistencies that have been reported regarding the ability of TFIID to activate Sp1-driven promoters (Smale et al., 1990). If binding of Sp1 is absolutely required for activity of a promoter, it cannot be transcribed in the 47°C heat-treated extract simply by the addition of TFIID. However, if Sp1 is just one of several activator proteins utilized by a promoter, then it may be possible to restore activity for that promoter by addition of TFIID to 47°C heat-treated extracts. Second, our results suggest that studies of the transcriptional regulation of Sp1-activated promoters should not be performed at 30°C (the temperature at which many investigators perform transcription reactions) because at least one important component of the complex (Sp1 binding) will be inactivated. Our results explain the previously reported observation that DHFR (Farnham and Schimke, 1986) and CAD (Farnham and Kollmar, 1990) transcription is optimal at temperatures lower than 30°C. We have also confirmed that transcription from the REP and CMV promoters is greater at 24°C than at 30°C. It is likely that many Sp1-activated promoters will have similar temperature optima.

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