The EMBO Journal Vol.17 No.11 pp.3146–3154, 1998

Transcriptional activation domains stimulate initiation and elongation at different times and via different residues

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Transcriptional activators can stimulate multiple steps in the transcription process. We have used GAL4 fusion proteins to characterize the ability of different transcriptional activation domains to stimulate transcriptional elongation on the *hsp70* gene *in vitro*. Stimulation of elongation apparently occurs via a mechanistic pathway different from that of stimulation of initiation: the herpes simplex virus VP16, heat shock factor 1 (HSF1) and amphipathic helix (AH) activation domains all stimulate initiation, but only VP16 and HSF1 stimulate elongation; and mutations in hydrophobic residues of the HSF1 activation domains impair stimulation of elongation but not of initiation, while mutations in adjacent acidic residues impair stimulation of initiation more than of elongation. Experiments in which activators were exchanged between initiation and elongation demonstrate that the elongation function of HSF1 will stimulate RNA polymerase that has initiated and is transcriptionally engaged. Transcriptional activators thus appear to have at least two distinct functions that reside in the same domain, and that act at different times to stimulate initiation and elongation.

Keywords: heat shock/nucleosomes/transcriptional activation/transcriptional elongation

Introduction

Both prokaryotic and eukaryotic transcription often require the actions of gene-specific activators to stimulate specific steps in the transcriptional process. In the majority of eukaryotic cases tested, such activators display the ability to stimulate the first step in the transcription process, the recruitment of general factors to form a pre-initiation complex (reviewed in Ptashne and Gann, 1990; Tijan and Maniatis, 1994). Nevertheless, numerous examples of regulated transcriptional elongation exist in both prokaryotes and eukaryotes (reviewed in Spencer and Groudine, 1990; Greenblatt *et al*., 1993). What types of activators might achieve this regulation, and how they do so, remains largely enigmatic.

One of the best characterized examples of regulated elongation in eukaryotes is the *hsp70* heat shock response gene. Both human and *Drosophila hsp70* genes exhibit a regulatory block to transcription near their 5′ ends. On the uninduced gene, a paused, transcriptionally engaged RNA polymerase ternary complex is present over a narrow region centered at +21 to +35 in *Drosophila* (Rougvie and Lis, 1988; Giardina *et al*., 1992; Rasmussen and Lis, 1993) and at +45 in humans (Brown *et al*., 1996). In response to heat shock, not only does the rate of initiation increase, but the transit time of polymerase through the pause is drastically reduced.

In humans, the *hsp70* gene is regulated by the heat shock factor 1 (HSF1) transcriptional activator. *In vivo*, the binding of this factor to its elements in the *hsp70* promoter is necessary for gene activation (Pelham, 1982). *In vitro*, this activation consists of increasing both the template utilization or rate of initiation (Rabindran *et al*., 1991) and read-through of the pause site at +45 (Brown *et al*., 1996). Transfection studies with HSF chimeras have localized the domains of HSF1 that possess activation activity to amino acids 371–529 (Green *et al*., 1995); however, the characteristics of these domains that allow them to activate transcription are poorly understood.

Mutational analyses of the VP16 and HSF1 activation domains have begun to delineate the types of amino acids important for activation. Specific acidic and hydrophobic residues are essential for the transcriptional activity of these proteins *in vivo* (Cress and Triezenberg, 1991; Regier *et al*., 1993; Newton *et al*., 1996). Similarly, previous investigators have shown that some activators, like GAL4–VP16 and GAL4–E1a, can stimulate transcriptional elongation on transfected or injected reporter genes better than others such as GAL4–AH or GAL4–SP1 (Yankulov *et al*., 1994; Blau *et al*., 1996). How these activators increase elongation remains unknown.

We wished to explore in more detail how the same HSF1 transcriptional activator can stimulate both initiation and elongation. It has been known for some time that activators can stimulate initiation by promoting the formation of active transcription complexes at the promoter. We have employed an *in vitro* elongation assay to separate the steps of transcription. We use this protocol to show that different amino acids in the HSF1 activation domain are required for stimulation of elongation and initiation, and that the elongation function of HSF1 works late, after RNA polymerase has initiated and cleared the promoter.

Results

Activators differ in their ability to stimulate elongation in vitro

We have shown previously that the HSF1 activation domains can stimulate elongation on the *hsp70* gene

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in vitro (Brown *et al*., 1996). To begin to understand the properties of HSF1 that allow it to do this, we examined different transcriptional activation domains for their ability to stimulate elongation in the same *in vitro* system. This system employs polystyrene bead-tethered templates to control precisely the nucleotide content of a transcription reaction, and thereby to separate the steps of transcription initiation from those of elongation. When the human *hsp70* gene is used as a template in this system, it mimics the promoter-proximal transcriptional pausing observed on this gene *in vivo* in a chromatin-dependent fashion, and HSF1 can stimulate elongation through this pause (Brown *et al*., 1996).

To look at the effects of different transcriptional activation domains upon elongation in this system, we used the following procedure. The template was a human *hsp70* gene in which the upstream heat shock elements have been replaced by five GAL4 DNA-binding sites, and in which four point mutations were engineered to eliminate guanosine nucleotides in the first 15 bases of the transcribed region. The entire template was tethered to polystyrene beads via a biotin–streptavidin linkage to facilitate changes of nucleotides and factors. Transcription was initiated on this template using HeLa basic transcription factors (partially purified to remove contaminating nucleotides), radiolabeled UTP and low concentrations of ATP and CTP. Under these 'G-less' conditions, templates were generated that contain RNA polymerase artificially stalled at +15 with a labeled nascent transcript. After nucleotides and loosely bound proteins were washed away from these templates with 1% Sarkosyl detergent, the template was assembled into nucleosomes and elongation was permitted to continue in the presence of all four unlabeled nucleotides. Nucleosome assembly is required in order to see an extended pause, and in order for activators to stimulate elongation (Brown *et al*., 1996).

Two types of information are obtained using this protocol. The ratio of full-length transcripts to transcripts paused at +46 to +49 provides a measure of the efficiency of transcriptional elongation. In addition, the reaction conditions used here do not permit reinitiation, and thus the total number of transcripts in a lane is a measure of the number of templates that are able to support productive transcription initiation and promoter clearance (i.e. template utilization).

As observed previously (Brown *et al*., 1996), when transcriptional activators were absent from the reactions, most RNA polymerase remained stably paused at 46–49 bases from the start of transcription, even after an hour in the presence of high concentrations of nucleotides (Figure 1A, lanes 1, 4, 7 and 10). If a 6-fold molar excess of human HSF1 transcriptional activation domains fused to the GAL4 DNA-binding domain was present, full-length transcript was also made (Figure 1A, lane 9). No stimulation of elongation was observed with the GAL4 DNA-binding domain alone (Figure 1A, lanes 10–12). Interestingly, a 1.5-fold excess of HSF1 was able to increase the total transcription in a reaction, and hence template utilization, but was unable to stimulate elongation significantly, as the paused transcripts and the full-length transcript both increased to a similar degree (Figure 1A, lane 8; see below).

To understand how different activation domains behave in this system, we compared the abilities of two other heterologous activators, the amphipathic helix (AH) of Giniger and Ptashne (1987) and the herpes simplex virus VP16 activation domain (Triezenberg *et al*., 1988), to stimulate elongation and total transcription. Whereas a

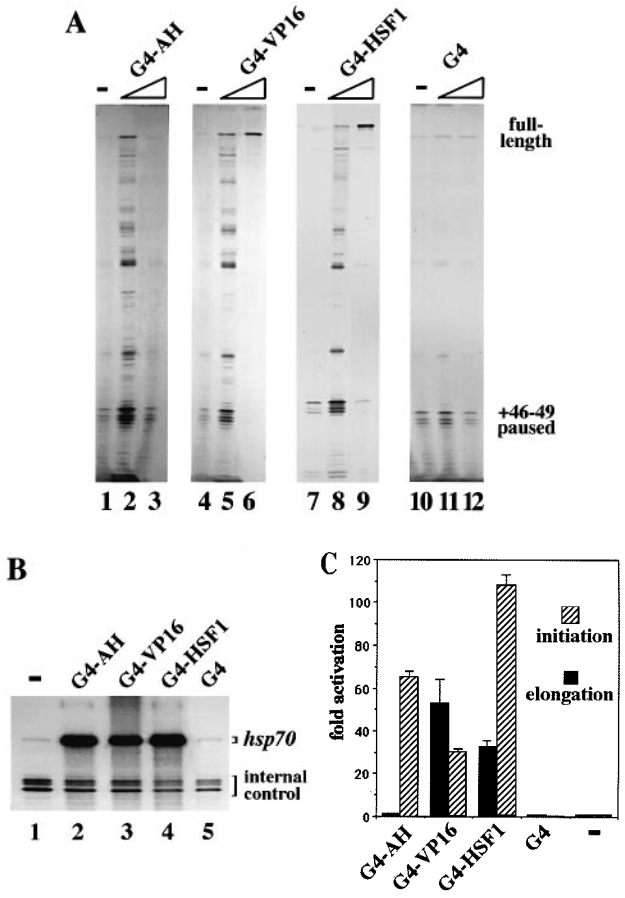

Fig. 1. (**A**) Effects of different activators upon elongation. Transcription complexes were stalled at +15 on human *hsp70* template pSAB12 (containing five GAL4 DNA-binding sites and a short G-less region from +1 to +15), washed, the template was assembled into nucleosomes and the complexes were elongated. During these reactions, either no activator (lanes 1, 4, 7 and 10), the GAL4(1–147) DNA-binding domain (lanes 11 and 12), G4–AH (lanes 2 and 3), G4–VP16 (lanes 5 and 6) or G4–HSF1 (lanes 8 and 9) was present. Activators were present at either a 1.5-fold (lanes 2, 5, 8 and 11) or a 6-fold excess (lanes 3, 6, 9 and 12) relative to binding sites. (**B**) Effects of different activators upon total transcription. Transcription was carried out on *hsp70* template pG5HC2AT (containing five GAL4 sites and a 380 bp G-less cassette). During these reactions, either no activator was present (lane 1), or a 1.5-fold molar excess of G4–AH (lane 2), G4–VP16 (lane 3), G4–HSF1 (lane 4) or G4 (lane 5). Transcription products were compared with transcription from plasmid pΔ53short (containing no GAL4 sites) present in the same reactions. (**C**) Quantitation of the effects of different activators upon elongation and upon total transcription. Elongation was quantitated as the ratio of full-length transcripts to transcripts paused at +46 to +49 using the assay described in (A). Numbers were expressed relative to the amount of elongation in the absence of activator. Total transcription was quantitated as the amount of transcription from the *hsp70* promoter using the assay described in (B). It was normalized to the amount of transcription from the pΔ53short internal control, and expressed relative to the amount of transcription in the absence of activator. Over three trials, relative activation strengths but not necessarily absolute activation by each activator were entirely consistent. Numbers are plotted with standard errors and average values from these three trials.

6-fold molar excess of GAL4–VP16 stimulated read-through of pausing as well as GAL4–HSF did (Figure 1A, lane 6), GAL4–AH had no effect upon elongation (Figure 1A, lane 3). In contrast, at a 1.5-fold molar excess, these two activators increased the total signal in each lane to comparable extents (Figure 1A, lanes 2 and 5), suggesting that they had equivalent effects upon the steps of initiation. The relative abilities of VP16 and AH to stimulate initiation in our assay are consistent with previous *in vitro* investigations. Although VP16 is a much stronger activator than AH *in vivo*, the two activate transcription comparably *in vitro* on naked DNA templates in pre-binding protocols; however, VP16 is more potent than AH in these protocols when templates are assembled with nucleosomes (Workman *et al*., 1991; this study).

The protocol described above provides a direct comparison between the ability of an activator to stimulate elongation and its ability to stimulate template utilization. This comparison is qualitative in nature because it is not possible to quantify template utilization accurately, as that would require summing all of the numerous transcripts in a given lane. To provide a second measure of the ability of activators to stimulate transcription at steps other than elongation that is readily quantified, we performed transcription reactions using naked DNA templates. We have shown previously that activators do not stimulate either the rate or efficiency of elongation on the naked hsp70 gene *in vitro* (Brown *et al*., 1996). Therefore, stimulation by activators in this assay might measure effects upon general transcription factor recruitment, pre-initiation complex formation, initiation, promoter clearance or reinitiation; for simplicity, we will refer to these steps collectively as 'initiation'. Using this assay, the HSF1, VP16 and AH activation domains stimulated transcription of the *hsp70* promoter to roughly the same degree (Figure 1B, lanes 2–4), but the GAL4 DNA-binding domain alone did not stimulate transcription (Figure 1B, lane 5). Qualitatively identical results were seen above using the single-round protocol that provided a measure of template utilization. Hence, we concluded that the abilities of these transcriptional activation domains to stimulate initiation did not correlate with their ability to stimulate elongation. The effects of different activators upon elongation (Figure 1A) and initiation (as in Figure 1B) were measured several times and are quantitated in Figure 1C. The trends observed for multi-round 'initiation' experiments were identical to those observed in single-round 'template utilization' experiments (Figure 2; data not shown).

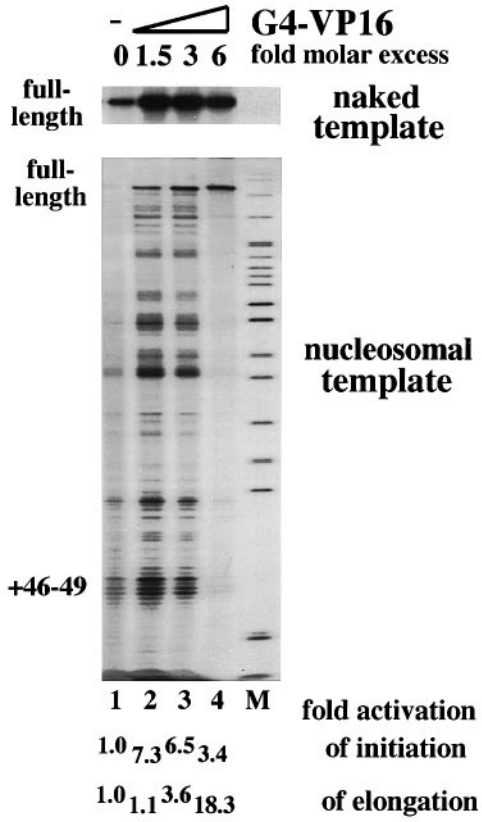

Fig. 2. Comparison of the activation of elongation and total transcription by G4–VP16. Elongation reactions were carried out by stalling RNA polymerase at +15 on template pSAB12, either assembling (bottom panel) or not assembling (top panel) the template into nucleosomes, and continuing elongation. During these reactions, G4–VP16 was either not present (lane 1) or present at a 1.5-fold (lane 2), 3-fold (lane 3) or 6-fold (lane 4) molar excess. Relative initiation was taken as the amount of total transcription in the top panel, and quantitated relative to transcription in the absence of activator. The read-through ratio in each reaction in the bottom panel was quantitated as the ratio of the amount of full-length transcript to the amount of +46 to +49 paused transcript.

Different concentrations of activators are required to stimulate initiation and elongation maximally at hsp70

The fact that different concentrations of activator stimulate initiation and elongation maximally has already been noted in Figure 1A. This effect is demonstrated more graphically in the titration of Figure 2. In the top panel, the single-round protocol was used with naked DNA templates so that all transcripts reached full length. In this case, a 1.5-fold molar excess of GAL4–VP16 relative to DNA-binding sites stimulated template utilization maximally (Figure 2, top panel, lane 2). The same trend can be verified by looking at the total transcripts in the bottom panel (Figure 2, lane 2), in which the standard elongation assay used in Figure 1 was employed. By contrast, very little stimulation of read-through is observed at this concentration of activator (Figure 2, ratio of full-length transcript to paused in bottom panel, lane 2). Maximal effects of activator upon elongation through the *hsp70* pause site are not seen until a 6-fold excess of activator is present in the elongation reactions (Figure 2, lane 5). At this concentration of activator, the total amount of transcription in the lane has begun to decrease even as elongation is being stimulated maximally. These observations might reflect a difference in the mechanism used by activators to stimulate elongation and initiation; however, the observed inhibition of initiation at high activator concentration confounds a rigorous interpretation of these results, as we do not understand the mechanism of this inhibition.

Two different domains of human HSF1 stimulate both initiation and elongation

The HSF1 activation domains stimulate both transcriptional elongation and initiation (Figure 1). Previous investigations have demonstrated that transcriptional activation

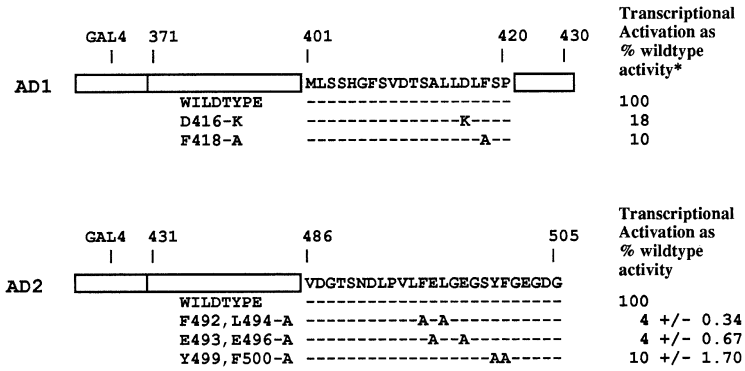

Fig. 3. Effects of mutations in human HSF1 AD1 and AD2 upon transcriptional activation in HeLa cells (*). Effects of AD1 mutations upon transcriptional activation are reproduced from Newton *et al.* (1996). Effects of AD2 mutations were measured as follows: plasmids expressing wild-type and mutated AD2 activation domains fused to the GAL4 DNA-binding domain were transfected into HeLa cells in the presence of p540CAT reporter (containing five GAL4 DNA-binding sites upstream of the human *hsp70* promoter fused to the CAT gene) and pXHG5 reference plasmid (expressing human growth hormone). CAT expression was measured and normalized to human growth hormone expression. Numbers are shown relative to the CAT expression induced by the wild-type AD2 domain. The standard errors shown are compiled from 4–10 trials with each mutant. Each construct was shown to have similar DNA-binding ability as measured by EMSA using nuclear extracts from the transfected cells (data not shown).

by HSF1 *in vivo* is localized to two distinct domains, activation domain I (AD1) spanning amino acids 371–430, and activation domain II (AD2) spanning amino acids 431–505 (Green *et al*., 1995; Shi *et al*., 1995; Zuo *et al*., 1995; Newton *et al*., 1996; Figure 3). We tested whether the two stimulatory abilities of HSF1 might be separated between these two domains. GAL4 fusion proteins containing each of these domains were purified and then were tested in the initiation and elongation assays described above to determine their abilities to stimulate the steps of initiation and elongation (Figure 4). When these activators were added to the multi-round transcription assay used in Figure 1B, they stimulated total transcription equivalently (Figure 4A, lanes 2 and 6). When they were added to the elongation assay used in Figure 1A, they increased both total transcription in the lane and the ratio of full-length to paused transcripts (Figure 4B, lanes 2 and 7). We conclude from these two assays that both activation domains of HSF1 can stimulate steps in both transcriptional initiation and transcriptional elongation.

Hydrophobic residues in HSF1 are important for the stimulation of elongation

We next tested whether mutations might be found in the two bifunctional HSF1 activation domains that reduce their ability to activate either initiation or elongation separately. To find such mutations, we first searched for mutations in the HSF1 activation domains that affect their ability to activate transcription of *hsp70 in vivo*, and then analyzed the effects of these mutations upon stimulation of initiation and elongation by HSF1 *in vitro*.

We constructed 10 double point mutations of a central 50 amino acid domain of AD2 highly conserved between humans and chickens; all mutations were made in non-proline residues that were identical between the two species. These mutant activation domains were fused to the GAL4 DNA-binding domain and transiently transfected into HeLa cells, where their ability to activate transcription from an *hsp70*–CAT reporter gene regulated via GAL4-binding sites was examined by measuring the level of CAT activity in extracts from these cells. Of these mutations, three reduced transcription >10-fold in this *in vivo* assay, and were chosen for *in vitro* characterization (Figure 3B). One of these double mutations changed acidic residues (Glu493 and Glu496 to Ala) and two changed bulky hydrophobic residues including one phenylalanine (Phe492 and Leu 494 to Ala, and Tyr499 and Phe500 to Ala). The locations of these mutations and their effects *in vivo* are summarized in Figure 3B.

A similar deletion and mutagenesis of AD1 previously found two single mutations in a 20 amino acid conserved core domain that display a 5- to 10-fold impaired ability to activate the *hsp70* promoter in transient transfections (Newton *et al*., 1996; see Figure 3A). One of these changed an acidic residue and one changed a phenylalanine (Asp416 to Lys and Phe418 to Ala).

To look at the effects of these mutations upon the ability of AD1 and AD2 to affect the steps of transcription initiation and elongation on *hsp70 in vitro*, the activation domains were expressed as bacterial fusions to the GAL4 DNA-binding domain, purified, and then used in the initiation and elongation assays described above. When each of the mutant proteins was compared with the wild-type proteins in the same assays, they displayed differing effects upon initiation and elongation.

The double point mutations in AD2 had greater effects in the *in vitro* assays than the single point mutations in AD1 (Figure 4A and B). Added to the multi-round initiation assay, mutants that were altered in the bulky hydrophobic residues of AD2 were not impaired in their ability to stimulate the steps of initiation (Figure 4A, compare lanes 7 and 8 with 6). These same mutations did impair the activation of elongation when they were added to an elongation assay, as can be seen by the decrease in full-length transcript relative to wild-type in each lane, even as the total transcription in each lane remained equivalent to that observed for the wild-type (Figure 4B, compare lanes 9 and 10 with 7). By contrast, mutation of two acidic residues in AD2 reduced the level of transcription in the multi-round assay (Figure 4A, lane 9), but had lesser effects upon elongation (Figure 4B, lane 11). Similar trends were seen with the single point mutations in AD1, although the effects, while reproducible, were modest. The acidic mutation decreased initiation more than the

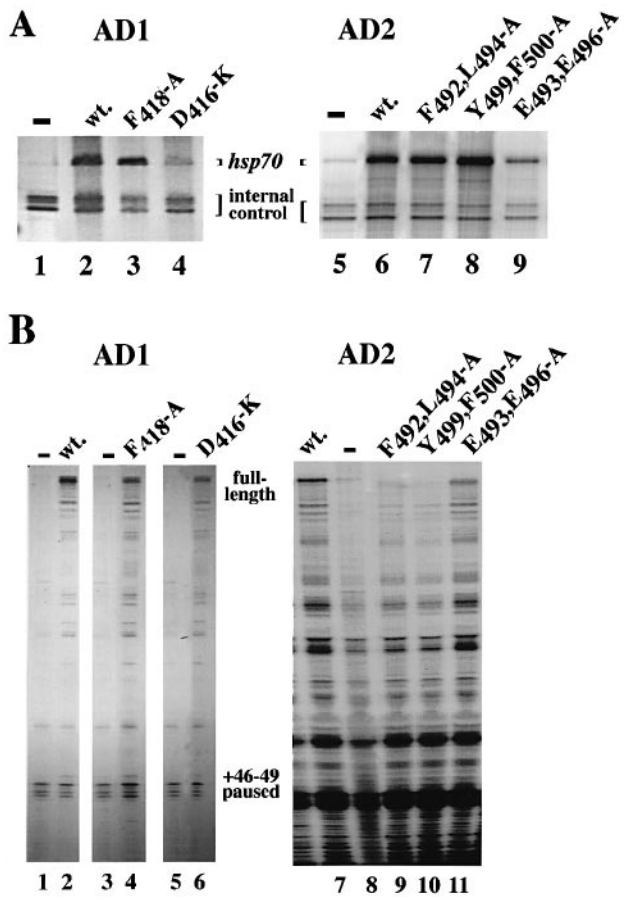

Fig. 4. Effects of mutations in HSF1 AD1 and AD2 *in vitro*. (**A**) Effects of mutations upon total transcription. The assay described in Figure 1B was used to measure the ability of G4–AD1, G4–AD2 and mutants of them to activate transcription in the absence of nucleosomes. Reactions contained either no activator (lanes 1 and 5) or a 1.5-fold molar excess of activator. (**B**) Effects of mutations upon elongation. The assay described in Figure 1A was used to measure the ability of G4–AD1, G4–AD2 and mutants of them to stimulate elongation. Two different experiments are shown for AD2 mutants. Reactions contained either no activator (lanes 1, 3, 5 and 7) or a 6-fold molar excess of G4–AD1 (lane 2), G4–AD1 mutant F418-A (lane 4), mutant D416-K (lane 6), G4–AD2 (lane 8), mutant F492,L494-A (lanes 9 and 12), mutant Y499,F500-A (lanes 10 and 13) or mutant E493,E496-A (lanes 11 and 14).

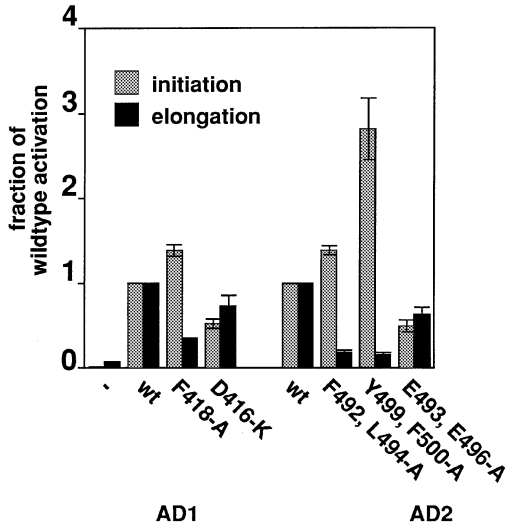

Fig. 5. Abilities of HSF1 AD1, AD2 and mutants of them to stimulate initiation and elongation *in vitro*. The stimulation of total transcription and of elongation by G4–AD1 and G4–AD2 mutants is plotted relative to the stimulatory ability of the wild-type proteins. Results shown are the cumulative averages and standard errors from three (AD1) or four trials (AD2). The errors are based on relative strengths of activation domains from experiment to experiment, which were quite consistent; the absolute level of activation varied modestly from trial to trial. Elongation and total transcription initiation were quantitated as described in Figure 1C.

hydrophobic mutation (Figure 4A), while the hydrophobic mutation had a greater effect on elongation than the acidic mutation (Figure 4B, compare the ratios of full-length to paused).

The effects of these different mutations are presented graphically in Figure 5. From this, we can conclude that adjacent or even interspersed mutations in the two activation domains have different effects upon the steps of initiation and elongation. Bulky hydrophobic residues, in particular, appear to play a role in elongation.

Activators present only during elongation can stimulate elongation through nucleosomes

Based on the experiments above, it is likely that the activation of initiation and elongation are distinct processes. An equally important question, not previously addressed, is that of when activators act to stimulate elongation. Activators added either during the steps of initiation or during the steps of elongation conceivably could affect elongation. The *in vitro* elongation assay that we have developed allows us to add activators during the steps of initiation, wash them away with Sarkosyl detergent after initiation has occurred, and then continue elongation in their absence, or vice versa. In this way, we can answer precisely the question of when activator is required to stimulate elongation.

Experiments that address this issue are shown in Figure 6. We added a 4.5-fold molar excess of GAL4–HSF1 to transcription reactions either during initiation, during elongation or at both times, and looked at its ability to facilitate read-through of pausing under each circumstance. When HSF1 was present only during the steps of initiation, no stimulation of read-through was observed, but the total transcription in the lane increased (Figure 6, lane 2). When HSF1 was present throughout the reaction, read-through was increased, as well as the total amount of transcription (Figure 6, lane 3; previous figures). By contrast, when HSF1 was added after initiation of transcription and template assembly into nucleosomes, so that it was present only during elongation, it still possessed the ability to facilitate read-through of pausing (Figure 6, lane 4), though now no stimulation of total transcription was observed. Therefore, activators could act specifically during elongation to stimulate existing transcription complexes to traverse the pause site at *hsp70*.

Further evidence that activators are able to stimulate elongation independently of earlier transcriptional steps is provided by 'swapping' experiments in which the HSF1 activation domain is present during initiation and then traded for the AH domain during elongation, or vice versa (Figure 6B). Since Figure 1 showed that both activation domains could stimulate the steps of initiation but only HSF1 could stimulate elongation, we would predict that only when HSF was present would elongation be stimu-

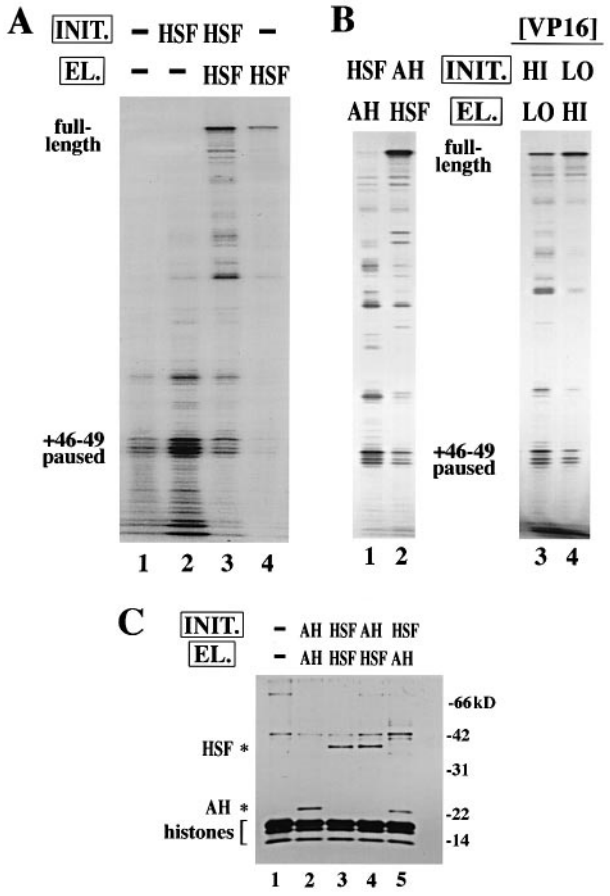

Fig. 6. Time of activator addition. (**A**) The ability of the G4–HSF1 activator to stimulate elongation on template pSAB12 was examined in a standard elongation reaction: transcription complexes were stalled at +15, templates were assembled into nucleosomes and elongation was continued. G4–HSF1 was either not present (lane 1), or present at a 4.5-fold molar excess during initiation only (lane 2), throughout the reaction (lane 3) or during elongation only (lane 4). (**B**) The ability of different activators or different concentrations of the same activator to stimulate initiation and elongation during different steps of the elongation reaction was tested. The standard elongation reaction described in (A) was repeated for the left panel, but either G4–HSF1 was present during initiation and G4–AH during assembly and elongation (lane 1), or G4–AH was present during initiation and G4–HSF1 during assembly and elongation (lane 2). The standard elongation reaction was again repeated for the right panel, but this time either a 6-fold molar excess of G4–VP16 was present during initiation and a 1.5-fold molar excess during assembly and elongation (lane 3), or a 1.5-fold molar excess of G4–VP16 was present during initiation and a 6-fold excess during elongation (lane 4). (**C**) SDS–PAGE analysis of proteins in transcription reactions. Elongation reactions were carried out using a procedure identical to that used in the left panel of (B), except that reactions were scaled to twice the size and, at the conclusion of elongation, bead-bound templates were rinsed, run on an SDS–polyacrylamide gel and silver-stained to examine the proteins bound to them. During the transcription reactions themselves, either no activator was added (lane 1), a 4.5-fold molar excess of G4–AH (lane 2) or G4–HSF1 (lane 3) was present throughout, or G4–AH was present during initiation and G4–HSF1 during assembly and elongation (lane 4), or G4–HSF1 was present during initiation and G4–AH during assembly and elongation (lane 5).

lated. This result can be seen in Figure 6B. When GAL4–HSF1 was present during initiation and GAL4–AH during elongation, then read-through of pausing was not observed (Figure 6B, lane 1). By contrast, if the order was reversed so that AH was present during initiation and HSF1 was present during elongation, read-through was seen (Figure 6B, lane 2).

A similar experiment can be performed by varying the concentrations of activator present during initiation and elongation. Figure 2 demonstrated that a 1.5-fold molar excess of VP16 could stimulate the steps of initiation better than those of elongation, whereas a 6-fold molar excess stimulated elongation maximally but initation less. Hence, we would predict that if a high concentration of GAL4–VP16 were added during initiation, followed by a low concentration during elongation, the observed stimulation of elongation would not be as great as when a high concentration of VP16 was added during elongation and a low concentration during initiation. This experiment is also shown in Figure 6B. When a high concentration of GAL4–VP16 was present during initiation, but only a modest concentration during elongation, little stimulation of elongation occurred (Figure 6B, lane 3). When this order was reversed so that a high concentration of VP16 was present during elongation, read-through was again observed (Figure 6B, lane 4). In these 'swapping' experiments, we could verify in each case that complete exchange of factors had occurred by loading reactions onto an SDS–PAGE gel at the end of the transcription reaction and silver staining to visualize the proteins present. One such gel, showing complete exchange of GAL4–AH and GAL4–HSF, is shown in Figure 6C. Hence we can conclude that activators can indeed stimulate elongation even when present only during elongation.

Discussion

In the experiments above, we have found that bulky hydrophobic residues within the HSF1 activation domains play a crucial role in their ability to stimulate elongation. These domains are able to stimulate already engaged RNA polymerases to elongate through a promoter-proximal pause on the *hsp70* gene. Other residues within these same domains contribute to their separate ability to stimulate earlier steps in transcription. Hence, the same HSF1 activation domains possess multiple abilities to act upon different steps of transcription at different times.

Different activation domains stimulate elongation to different degrees

Other investigators have demonstrated that different activators, when injected or transfected into cells, have unequal effects upon transcriptional elongation on *myc* or human immunodeficiency type 1 (HIV-1) reporter constructs (Yankulov *et al*., 1994; Blau *et al*., 1996). They demonstrated *in vivo* that the AH activation domain is less effective than VP16 in stimulating elongation, and that a quadruple point mutation in the phenylalanine residues of VP16 reduced its ability to stimulate elongation. From their *in vivo* assays, they were unable to determine whether the stimulation of elongation that they observed was due to the suppression of pausing or the suppression of termination on their reporter genes.

We have also found that VP16 but not AH can stimulate elongation, and that phenylalanine mutations in a different activator, HSF1, reduce its ability to stimulate elongation. The results obtained in the *in vitro* system employed in this work therefore correlate well with *in vivo* analyses of

activator function. Meanwhile, the nature of our *in vitro* experiments allowed us to address the mechanistic aspects of the regulatory process which are difficult to determine *in vivo*.

Activators stimulate elongation on hsp70 by acting upon polymerases that are already stalled

One question we were able to address is that of when an activator can function to stimulate elongation. It might exert its influence as the basal transcription factors are forming a complex on a given gene, thereby stimulating the formation of a 'processive' complex capable of elongating without premature pause or termination. Alternatively, it might act after polymerase complexes have already formed and paused, allowing them to bypass the blockage. Finally, it might act when polymerase complexes are newly initiated, allowing them to read through subsequent pause or termination sites. Any combination of the above mechanisms is also possible.

Factors employing each of these mechanisms have been identified previously. The latter mechanism, in which activators act upon an already initiated transcription complex to allow it to bypass subsequent pause or termination signals, is employed by both the bacteriophage λ N protein and the HIV Tat protein (reviewed in Cullen, 1990; Das, 1992). By contrast, both the bacteriophage λ Q protein and the eukaryotic elongation factor SII can act upon paused polymerase complexes to restart them and/or to prevent subsequent pausing (Rappaport *et al*., 1987; Reines *et al*., 1989; Yang and Roberts, 1989). Finally, factors like P-TEFb and TFIIH appear to play a role in the formation of productive elongation complexes *de novo* at eukaryotic promoters (Goodrich and Tjian, 1994; Marshall *et al*., 1996; Zhu *et al*., 1997).

Because our *in vitro* elongation assay allowed us to control which steps in transcription were conducted in the presence of activator, we were able to conclude that in order to stimulate elongation, activators had to be present after the initiation of transcription had occurred and polymerase had been stalled. Hence, our experiments suggest that activators act on *hsp70* while polymerase complexes are paused to allow them to traverse this pause. Previous investigations from a number of laboratories are consistent with the hypothesis that RNA polymerase remains paused on the *hsp70* gene because of interactions with upstream factors and because of the inhibitory effects of chromatin upon transcriptional elongation. Hence, to stimulate elongation of paused polymerases, activators might affect the interactions of the paused RNA polymerase with transcription factors remaining at the promoter (Lis and Wu, 1993; Purnell *et al.*, 1994). Alternatively, they might stimulate the remodeling of downstream chromatin structure (Lis and Wu, 1993; Brown *et al*., 1996), for example by recruiting either ATP-dependent remodeling complexes and/or acetyltransferases.

Multiple distinct steps are stimulated by the same HSF1 activation domains

Our experiments have demonstrated that the two activation domains of human HSF1 are both able to stimulate steps in transcriptional initiation and transcriptional elongation. Mutations of bulky hydrophobic residues in the two different HSF1 activation domains affected their ability to stimulate elongation but not initiation. We also tested adjacent and even interspersed mutations of acidic residues, and found that they had more significant effects on initiation than on elongation. In addition, higher concentrations of activator were required to stimulate elongation than were required to stimulate initiation. It is possible that higher concentrations of activator are needed simply to increase activator occupancy on nucleosomal templates. Alternatively, excess activator might be needed to titrate away a specific inhibitor of the elongation reaction, or to help break initiation-specific interactions between promoter-bound activator and general transcription factors, thereby allowing elongation-specific interactions to occur also.

Either way, the simplest model to explain all of these observations is that a single activation domain may interact with separate targets at different times to stimulate initiation and elongation. Hydrophobic residues play a role in interactions that facilitate elongation.

Materials and methods

Mutagenesis and expression of HSF1 AD2

The sequence coding for amino acid residues 431–505 of human HSF1 was subcloned into pBluescript SK(+) and transformed into *dut*$^{-}$*ung*$^{-}$ *Escherichia coli* strain R382 to produce single-stranded uracil-substituted plasmid, using M13K07 helper phage (Pharmacia). Point mutations were produced by modified Kunkel mutagenesis as described in Newton *et al.* (1996). These mutants were subcloned into the pBXG1 mammalian expression vector, which encodes the first 147 residues of the yeast GAL4 protein (i.e. the DNA-binding domain) followed by a multiple cloning site. (The vector is a gift of M.Ptashne, Harvard University.)

To verify that the expression levels of AD2 and mutants in these vectors were equivalent (data not shown), the vectors were transfected into HeLa cells and GAL4–AD2 activity was measured by electrophoretic mobility shift assay in cellular extracts (Newton *et al*., 1996).

Transient transfections and CAT assays

HeLa cells were grown and transfections were carried out as described by Newton *et al*. (1996), with 5 μg of pBXG1–AD2 plasmid expressing GAL4–HSF1 AD2 or GAL4–mutant AD2, 5 μg of p540CAT reporter plasmid and 1 μg of pXHG5 reference plasmid. Plasmid p540CAT contains five GAL4-binding sites at position –40 of the *hsp70* basal promoter. This promoter is fused at position +165 to the coding region of the CAT gene. Plasmid pXHG5 carries the human growth hormone (HGH) gene controlled by the mMT-1 promoter.

Cells were harvested and CAT expression was measured by phase extraction assay (Ausubel *et al*., 1989). The CAT assays were carried out for 1 h in 100 μl at 37°C with 25 μg of *n*-butyryl-coenzyme A and 0.2 μCi of [^{3}H]chloramphenicol (32 Ci/mmol; New England Nuclear-Dupont). Reactions were stopped by extraction with 200 μl of 2:1 TMPD-xylenes, and 150 μl of the organic phase was removed to scintillation fluid for quantitation. CAT activities obtained were normalized to HGH expression and assayed using the HGH Transient Gene Expression System kit (Nichols Institute Diagnostics).

Templates for in vitro transcription

For all *in vitro* elongation reactions, the template used was linearized pSAB12 tethered to avidin–polystyrene beads (Brown *et al*., 1996). It contains five GAL4 DNA-binding sites upstream of human *hsp70* promoter sequences from –34 to +567. Four point mutations were engineered to eliminate guanosine residues between +1 and +14.

For all multi-round initiation reactions, the template used was pG5HC2AT (Workman *et al*., 1991). It contains the same promoter as pSAB12, fused to a 380 bp synthetic cassette completely lacking guanosine nucleotides. The reference template in these reactions was pΔ53short (Sawadogo and Roeder, 1985). It contains the adenovirus ML promoter from –53 to +10, fused to a shorter G-less cassette of 300 nucleotides.

Activators and transcription factors used in transcriptions

The GAL4 DNA-binding domain (amino acids 1–94) and GAL4–VP16 and GAL4–AH fusion proteins were purified from *E.coli* as described

in Chasman *et al*. (1989). The former was 17 μM in concentration of dimer active for DNA binding, and was 80% pure and 80% active relative to total protein. GAL4–AH was 102 μM in active dimer, and was 45% pure and 100% active. GAL4–VP16 was 10 μM in active dimer, and was 50% pure and 90% active.

GAL4–HSF and mutants of it contained amino acids 1–147 of the GAL4 protein fused to various portions of the human HSF1 transcriptional activation domains, and were purified from *E.coli* as His_6-tagged fusion proteins by subcloning them into the pRJR1 expression vector (Reece *et al*., 1993) and purifying them over a nickel–Sepharose column (Qiagen) according to the manufacturer's directions. GAL4–HSF1 contained amino acids 370–529 of the HSF1 protein. The resulting preparation was 1.5 μM in concentration of active dimer, and was 90% pure and 80% active. GAL4–AD1 and mutants contained amino acids 371–430 of the wild-type HSF protein. The purified GAL4–HSF and GAL4–$HSF(D_{416}–K)$ proteins were 1.3 μM in active dimer, and the GAL4–$HSF(F_{418}–A)$ protein was 2.3 μM. All three preparations were ~50% pure and 85% active. GAL4–AD2 constructs contained amino acids 431–505 of HSF1. They were 3–4 μM in active dimer, and were 50% pure and 70% active. All proteins were dialyzed into buffer D [100 mM KCl, 20% glycerol, 20 mM HEPES pH 7.9, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF)].

Transcription factors, purified HeLa core histones, *Xenopus* nucleosome assembly extract and hSWI/SNF fractions were made as described in Brown *et al*. (1996).

In vitro elongation assays

Reactions were begun by pre-binding activators to their cognate DNA sites: we incubated 0.5 μg of bead-bound pSAB12 template for 15 min at room temperature in an 8 μl reaction including 2 mM $MgCl_2$ and a total of 4 μl of buffer D plus no activator, or a 1.5- to 6-fold molar excess of activator relative to DNA-binding sites, as specified. Transcription was initiated with partially purified HeLa transcription factors, RNA polymerase was artificially stalled at +15 by incubating with only ATP, CTP and [^{32}P]UTP nucleotides, templates were washed and nucleosomes were assembled with *Xenopus* assembly extract as previously described (Brown *et al*., 1996). In all reactions but those of Figure 6A and negative controls in other figures, activator was re-added after washing so that it was present during assembly as well. In Figure 6A, activator was not re-added during assembly. Reactions were then washed again, hSWI/SNF fractions were added to all reactions, and activator added where specified. Elongation was then continued with all four nucleotides, again as previously described (Brown *et al*., 1996). Completed reactions were phenol-extracted, EtOH-precipitated and then analyzed on a 7.5% acrylamide–7 M urea–1× TBE sequencing gel. Gels were exposed and quantitated on a Molecular Dynamics PhosphorImager.

Multi-round transcription assays

Transcription reactions were begun by pre-incubating a 1.5-fold molar excess of activator (relative to the concentration of binding sites) with 0.25 μg of plasmid pG5HC2AT and 0.25 μg of pΔ53short in an 8 μl reaction including 2 mM $MgCl_2$ and a total of 4 μl of buffer D plus activator. Reactions were then supplemented with 17 μl of a mix including 11 μl of transcription factors (as above), $MgCl_2$ and nucleotides, to make overall concentrations in the resulting 25 μl reaction equal to 2 mM $MgCl_2$, 0.6 mM ATP and CTP, 25 μM UTP, 0.5 μM [^{32}P]UTP (800 Ci/mmol, NEN) and 50 μM *O*-methyl-GTP. Each reaction also contained 50 U of RNase T1 (USB) and 15 U of RNasin (Promega). Reactions were allowed to incubate for 1 h at 30°C, and then stopped and analyzed identically to elongation reactions.

SDS–PAGE analysis of transcriptional elongation reactions

Transcriptional elongation reactions were performed exactly as described above, except that each reaction was twice the size. At the end of elongation, but prior to stopping reactions, they were washed once more with 0.6× buffer D to remove unbound proteins. Reactions were then pelleted again and resuspended in SDS sample buffer, and loaded onto a 13% polyacrylamide/0.25% bisacrylamide SDS gel. After electrophoresis, the gel was silver-stained according to the protocol of Wray *et al*. (1981).

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Received February 9, 1998; revised and accepted April 3, 1998