Activation of the TFIID–TFIIA complex with HMG-2

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The nonhistone chromosomal protein HMG-2 was identified as a factor necessary for activation in a defined transcription reaction in vitro containing RNA polymerase II and purified factors. Activation occurred on all promoters assayed except that of the immunoglobulin IgH gene. TFIIA was required for stimulated levels of transcription. The activation process depended on the presence of TAFs in the TFIID complex and generated a preinitiation complex from which TFIIB dissociated more slowly. However, titration of TFIIB over three orders of magnitude did not obviate the requirement of activator and HMG-2 to achieve stimulated levels of transcription. Analysis of the activated reaction identified the TFIID–TFIIA complex as the first stage of modification during activation. These results suggest that activation can occur solely in the presence of the basal factors, activator protein, and an "architectural" HMG factor, which probably stabilizes an activated conformation of the TFIID–TFIIA–promoter complex.

[*Key Words*: HMG-1/2; transcriptional activation; coactivator]

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The basal reaction for initiation of transcription by RNA polymerase II (Pol II) can be reconstituted with distinct protein factors that assemble on the promoter in an ordered fashion (for review, see Buratowski 1994). This process is nucleated by recognition of the TATA element by TFIID (or the TATA-binding protein, TBP) and culminates, after assembly, with conversion of the initiation complex into the elongation complex. The basal factors required for this reaction have been purified to homogeneity, and the genes for many have been cloned. On promoters such as the adenovirus major late (MLP), this set of factors is comprised of TFIID, TFIIB, TFIIE, TFIIF, TFIIH, and Pol II. The factors TFIIA and TFIIJ can stimulate the basal reaction (Samuels et al. 1982; Cortes et al. 1992).

Positive modulation of transcription requires additional activities, as reactions reconstituted with purified basal factors typically do not respond to activators such as SP1 and Gal4–VP16. Coactivating factors enable the basal factors to respond to sequence-specific activators. Stimulation of transcription in vitro is dependent on the TAF polypeptides that are associated with the TBP in TFIID (Dynlacht et al. 1991). Coactivators such as upstream stimulatory factor activity (USA) (Meisterernst et al. 1991) and ACF (Merino et al. 1993) are not associated with TBP. These coactivator fractions potentiate the stimulated reaction. Some of the components of these complex activities have been purified. The gene encoding PC4, a positive-acting component of USA, has been cloned recently. PC4 is thought to interact with both basal factors and sequence-specific activators (Ge and Roeder 1994a; Kretzschmar et al. 1994). Topoisomerase I also coactivates stimulation both by repression of basal and potentiation of stimulated transcription (Kretzschmar et al. 1993; Merino et al. 1993). It is possible that the high levels of activation in vivo are the result of combinations of potentiating and repressing factors (Meisterernst and Roeder 1991).

The degree of stimulation of transcription by activators is modest in reactions of defined basal factors, and this has made it difficult to determine the functional interactions of factors necessary for stimulation. In less defined systems, several of the basal factors have been implicated as targets of activation. Interactions of activator and TFIID fraction have been shown to yield both quantitative and qualitative effects in the binding of TFIID to the promoter (Sawadogo and Roeder 1985). The interaction of the TBP component of TFIID with an acidic activation domain (Stringer et al. 1990) has been proposed as a link in activation, as mutants in the VP16 activation domain that reduce function in vivo also reduce TBP association in vitro (Ingles et al. 1991). Functional assays using partially fractionated components have suggested TFIIB as a candidate target of activation (Lin and Green 1991; Choy and Green 1993). Recently, the activator Zta, in the presence of TFIIA, has been shown to enhance the rate of binding of TFIID to the promoter (Lieberman and Berk 1994). Additionally, the formation of the TFIID–TFIIA complex has been sug-

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gested to be a rate-limiting step in transcription facilitated by activator (Wang et al. 1992). Genetic and biochemical analyses in yeast suggest a different, important step in activation. Stimulation of transcription in vitro depends on the presence of a large Pol II complex composed of general transcription factors, suppressor of RNA polymerase B (SRB) proteins, and polymerase (Koleske and Young 1994; Kim et al. 1994).

Here we report the characterization and identification of a coactivator that functions in a defined transcription reaction, otherwise unable to respond to sequence-specific activators. The identification of the coactivator as high mobility group 2 (HMG-2), and the subsequent demonstration that the HMG box alone has the same function, suggest a structural role for these coactivators in establishing the activated initiation complex. The activation requires TFIIA, and an immobilized template transcription assay defines the TFIID–TFIIA complex as the first stage of initiation altered during activation.

Results

An activity potentiates activation: coactivator-B

Reconstitution of transcription with a defined set of basal factors (general transcription factors, GTFs) resulted in reactions that were not stimulated by the addition of sequence-specific activators. In these reactions, factors derived from cDNA clones were used where possible and the remaining purified components were defined reagents (see Materials and methods). Addition of each factor was absolutely required for transcription (Parvin and Sharp 1993; data not shown). The degree of stimulation was determined in reactions that contained both templates with and without binding sites for activators. Templates of the MLP with the activator-binding sites produced a 390-nucleotide transcript while the basal control template, which did not contain such binding sites, produced a 180-nucleotide message. This reconstituted reaction was active in the basal reaction but did not show significant stimulation upon addition of Gal4–VP16. However, we observed that addition of a MLTF (major late transcription factor) preparation derived from a heat-treated nuclear extract (Chodosh 1988) boosted transcription dramatically from templates containing five MLTF-binding sites (ML-5M) but not from basal control templates (ML) lacking these sites (data not shown). To determine whether the stimulation of transcription observed was mediated solely by MLTF or was the result of MLTF plus another activity, the MLTF preparation was subjected to conventional chromatography and fractions were assayed by transcription for MLTF stimulatory activity. MLTF separated from a coactivating activity that was subsequently purified to near homogeneity (see Materials and methods). This activity, called coactivator-B (Co-B), coeluted with a protein doublet of ~30 kD. To determine the generality of Co-B as a coactivator, it was tested with different sequence-specific activators and on different promoters.

Co-B functioned as a potentiator of stimulation with different classes of transcriptional activators. Addition of either the MLTF (Fig. 1A) or Gal4–VP16 (Fig. 1C) stimulated transcription from the appropriate template in the presence of Co-B (ML-5M and ML-3G respectively). Furthermore, the levels of stimulation with Co-B approached those observed in reactions containing nuclear extract (data not shown; Fig. 1B). Stimulation by SP1 was similarly affected by Co-B (data not shown). Co-B preparations were not contaminated with any basal factors, as they were unable to substitute for any of the these factors (data not shown), although titration of Co-B gave modest augmentation and then slight repression of the basal reaction (Fig. 1C, lanes 1–3). Co-B required the presence of both activator and activator-binding sites to exert its potentiation of stimulation. The activation observed in these reactions was attributable to a net increase in transcription from the specific template and was not the result of a selective derepression of the stimulated template nor a selective repression of the basal template (Fig. 1A, lanes 4–6).

To characterize the basal machinery requirements for activation, the necessity of TAFs in the TFIID complex

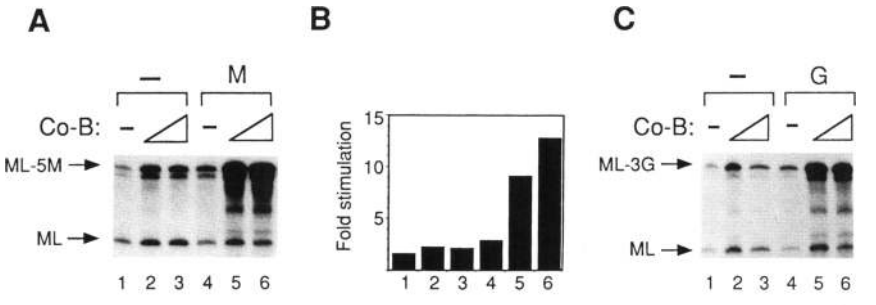

Figure 1. Ability of Co-B to stimulate transcription with different activators. (*A*) Activity with the cellular activator MLTF (M). ML transcript (180 nucleotides) was generated by 25 ng of supercoiled MLP basal control promoter. ML-5M transcript (390 nucleotides) was generated by 25 ng of supercoiled MLP with five MLTF-binding sites fused upstream. Transcripts were separated by urea–PAGE. (Lanes *1–3*) Titration of Co-B (0, 1, 5 μl) into the reaction in the absence of activator; (lanes *4–6*) titration of Co-B (0, 1, 5 μl) into the reaction in the presence of saturating amounts of MLTF (M). (*B*) Quantitation of stimulation from reactions shown in *A* was by PhosphorImager (Molecular Dynamics, Inc.). Fold stimulation is expressed as the ratio of message generated by ML-5M template vs. that generated by ML template. (*C*) Effect with the synthetic activator Gal4–VP16. ML transcript (180 nucleotides) was generated by 25 ng of supercoiled MLP basal control promoter. ML-3G transcript (390 nucleotides) was generated by 25 ng of supercoiled MLP with three Gal4-binding sites fused upstream. (Lanes *1–3*) Titration of Co-B (0, 1, 5 μl) into the reaction in the absence of Gal4–VP16; (lanes *4–6*) titration of Co-B (0, 1, 5 μl) into the reaction in the presence of saturating amounts of Gal4–VP16 (G).

was examined. In almost all in vitro systems tested, stimulation by activators is dependent on the presence of the holo-TFIID complex, which is composed of TBP and TAFs (Dynlacht et al. 1991; Zhou et al. 1992; for review, see Tjian and Maniatis 1994). This was also the case with Co-B; recombinant human TBP supported basal transcription but did not respond to Gal4–VP16 when Co-B was titrated into the reaction (Fig. 2A). The effect of Co-B on the basal reaction in the presence of TBP was similar to that observed with holo-TFIID: transcription increased slightly and then similarly decreased as Co-B was titrated into the reaction. Thus, the effect of Co-B on the basal reaction is TAF independent, whereas its effect on the stimulated reaction is critically dependent on TAFs. This implies that Co-B cannot obviate the need for TAFs in the stimulated reaction but, rather, functions as a complementing factor enabling the stimulatory effect of the activator on the basal machine.

The ability of Co-B to potentiate stimulation in the context of other promoters was tested. The μ-promoter of the heavy-chain immunoglobulin gene and the human heat shock promoter (HSP) were substituted for the MLP upstream of the 390-nucleotide G-less cassette. Gal4-binding sites were inserted upstream of the promoters (gifts of J. Parvin, Harvard Medical School, Boston, MA), and the templates were tested in the basal and stimulated reactions with the coactivator. The MLP without binding sites was included as a control promoter. The human HSP responded to activation potentiated by Co-B similarly to the MLP (data not shown). Surprisingly, Co-B exhibited a different effect on the μ-promoter (Fig. 2B). In the absence of Gal4–VP16, Co-B had opposing effects on the two promoters: transcription from the MLP increased slightly, as was observed in Figure 1, whereas transcription mediated by the μ-promoter decreased (lanes 1–3). When Gal4–VP16 was included in the titration, no activation was observed from the μ-promoter with its three Gal4-binding sites. However, at high levels of Co-B, the activator derepressed the μ-promoter and brought the transcription up almost to the basal level (cf. lanes 1, 3, and 6). This is in striking contrast to the MLP and HSP templates with Gal4–VP16-binding sites, which were strongly stimulated by activator as Co-B levels were increased, and suggests that the activation process potentiated by Co-B involves promoter-specific interactions of the initiation complex, coactivator, and the upstream activator (see Discussion).

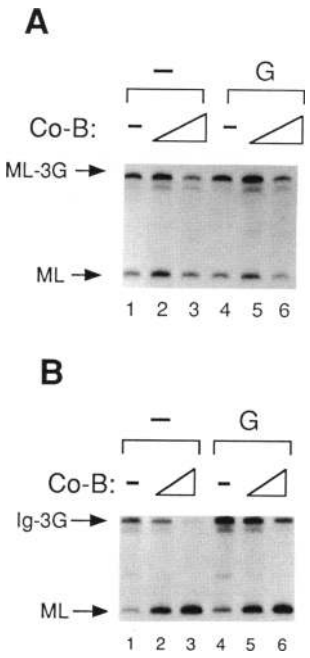

Figure 2. (*A*) Stimulation of activation by Co-B requires TAFs in the holo-TFIID complex. Titration of Co-B (0, 1, 5 μl) into transcription reactions with TBP in the absence (lanes *1–3*) and presence (lanes *4–6*) of Gal4–VP16. ML transcript (180 nucleotides) was generated by 25 ng of supercoiled MLP basal control promoter. ML-3G transcript (390 nucleotides) was generated by 25 ng of supercoiled MLP with three Gal4-binding sites fused upstream. (*B*) Template specificity of coactivator-B. Titration of Co-B (0, 1, 2 μl) into transcription reactions in the absence (lanes *1–3*) and presence (lanes *4–6*) of Gal4–VP16 (G). ML transcript (180 nucleotides) was generated by 25 ng of supercoiled MLP basal control template. Ig-3G transcript (390 nucleotides) was generated by 25 ng of supercoiled IgH μ-promoter with three Gal4 sites fused upstream.

Coactivator B is HMG-2

Reverse-phase high performance liquid chromatography (HPLC) was used to separate the two polypeptides that comigrated as ~30-kD bands in the Co-B preparation (Fig. 3A, lane 2). The HPLC fractions were resolved by SDS-PAGE and silver stained (Fig. 3A), and a portion of each fraction (A–J) was denatured with 6 M guanidine-HCl, renatured (see Materials and methods), and assayed by transcription in the presence of Gal4–VP16 (Fig. 3B). Fraction C, which contained the peak concentration of the larger polypeptide (Fig. 3A, lane 5) also contained the peak level of coactivator activity. (Fig. 3B, lane 5).

Amino-terminal protein sequencing of the HPLC-purified Co-B polypeptide generated a 21-amino-acid sequence that was identical to the amino-terminal sequence of the nonhistone chromosomal protein HMG-2. This nuclear protein also has a molecular mass of 30 kD in SDS-PAGE. To demonstrate that HMG-2 contained Co-B activity, recombinant HMG-2 was produced in bacteria from a human cDNA clone (Majumdar et al. 1991) and the protein was purified to homogeneity (see Materials and methods). The recombinant protein potentiated activation by Gal4–VP16 as efficiently as the original Co-B preparation (Fig. 4A). Typically, purified HMG-2 levels of 100 ng and levels of recombinant HMG-2 of 200–400 ng in the transcription reactions gave saturating amounts of activation, which suggests that only a few molecules of HMG-2 per template are required for activation (see Discussion). The modest stimulatory activity for basal transcription observed with the purified, cellular preparation was also observed with recombinant

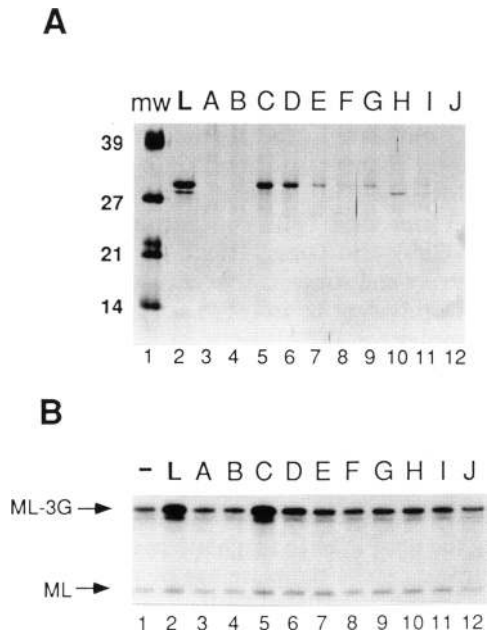

Figure 3. HPLC purification of Co-B and transcriptional activity of renatured fractions. (*A*) Purified Co-B preparation was subjected to reverse-phase HPLC using a C4 column, and fractions were resolved in a 14% SDS-polyacrylamide gel and silver stained. (Lane *2*) Load material; (lanes *3–12*) HPLC fractions *A–J*, respectively. (*B*) HPLC fractions and load were denatured with 6 M guanidium-HCl, renatured by dialysis, and assayed by transcription in the presence of Gal4–VP16. (Lanes *3–12*) HPLC fractions *A–J*, with load material (*L*) assayed in lane *2* and buffer added to reaction in lane *1*. ML transcript (180 nucleotides) was generated by 25 ng of supercoiled MLP basal control template. ML-3G transcript (390 nucleotides) was generated by 25 ng of supercoiled MLP with three Gal4-binding sites fused upstream.

HMG-2, indicating that this activity was not attributable to a contaminant.

HMG-1, the cellular homolog of HMG-2, is functionally equivalent in many assays in vitro (for review, see Grosschedl et al. 1994). This functional equivalence was also observed in the activation assay. Recombinant HMG-1 (generous gift of S. Lippard, Massachusetts Institute of Technology, Cambridge, MA) was titrated into basal (Fig. 4B, lanes 1–3) and Gal4–VP16-stimulated (lanes 4–6) transcription reactions. The coactivation by rHMG-1 suggested that a common feature of the HMG-1/2 family was responsible for such activity. The most striking common features of this family are subdomains that bind DNA nonspecifically in the minor groove. These subdomains, called HMG boxes, are repeated twice in HMG-1/2. The HMG box from the B-domain of HMG-1 is a folded polypeptide of ~80 amino acids that binds and dramatically bends DNA (Pil et al. 1993). A purified recombinant peptide corresponding to this domain was assayed in the reconstituted reaction and was functionally equivalent to HMG-1 (Fig. 4B, lanes 7–12). Thus, the sequences necessary for DNA binding by HMG-1/2 are also sufficient for coactivation of transcription.

The identification of HMG-1/2 as a coactivator of Pol II transcription in a defined in vitro system containing purified general transcription factors made it possible to study the mechanism of activation. The stimulated reaction was analyzed by titration of basal factors and by characterization of the effects of activation on individual steps in initiation.

TFIIA and TFIIB in activation

Consistent with previous observations (Ma et al. 1993), TFIIA augmented the basal reaction only slightly in the purified system used in these experiments. More recently, TFIIA was shown to be required, or to enhance in vitro, activation in systems of transcription factors of varying purity (Ozer et al. 1994; Sun et al. 1994; Yokomori et al. 1994). The requirement for TFIIA in activation potentiated by HMG-1/2 was tested (Fig. 5A). Titra-

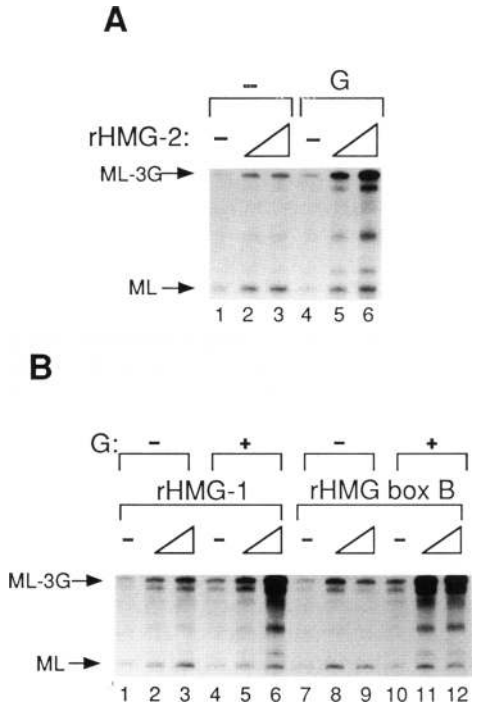

Figure 4. Recombinant HMG-1 and HMG-2 proteins coactivate stimulation. (*A*) Recombinant human HMG-2 protein was titrated into transcription reactions at 0, 100, and 500 ng, in the absence (lanes *1–3*) and the presence (lanes *4–6*) of Gal4–VP16 (G). ML transcript (180 nucleotides) was generated by 25 ng of supercoiled MLP basal control template. ML-3G transcript (390 nucleotides) was generated by 25 ng of supercoiled MLP with three Gal4-binding sites fused upstream. (*B*) Recombinant rat HMG-1 and recombinant HMG-1 B-subdomain were titrated into reactions in the absence (lanes *1–3* and *7–9*) and the presence (lanes *4–6* and *10–12*) of saturating amounts of Gal4–VP16 (G), at 0, 100, and 500 ng of HMG-1 and 0, 50, and 250 ng of the B-domain polypeptide. ML transcript (180 nucleotides) was generated by 25 ng of supercoiled MLP basal control template. ML-3G transcript (390 nucleotides) was generated by 25 ng of supercoiled MLP with three Gal4-binding sites fused upstream.

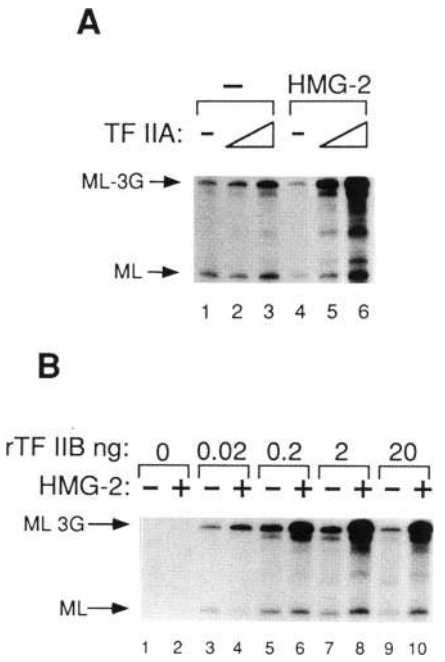

Figure 5. Roles of TFIIA and TFIIB in activation. (*A*) TFIIA is required for activation by Co-B. Homogeneous preparation of purified human TFIIA was titrated into transcription reactions at 0, 0.5, and 5 μl, in the absence (lanes *1–3*) and the presence (lanes *4–6*) of purified HMG-2 (pHMG-2). Gal4–VP16 was added to each reaction. ML transcript (180 nucleotides) was generated by 25 ng of supercoiled MLP basal control template. ML-3G transcript (390 nucleotides) was generated by 25 ng of supercoiled MLP with three Gal4-binding sites fused upstream. (*B*) Titration of rTFIIB. Recombinant human TFIIB was titrated into transcription reactions in the absence (odd-numbered lanes) and the presence (even-numbered lanes) of pHMG-2. Gal4–VP16 was added to each reaction. ML transcript (180 nucleotides) was generated by 25 ng of supercoiled MLP basal control template. ML-3G transcript (390 nucleotides) was generated by 25 ng of supercoiled MLP with three Gal4-binding sites fused upstream.

tion of TFIIA in the absence of HMG-1/2, but in the presence of activator, increased the level of transcription slightly from both templates with and without Gal4 sites (lanes 1–3). More interestingly, no stimulation was observed in the absence of TFIIA when HMG-1/2 and Gal4–VP16 were present (cf. lanes 1 and 4). To achieve significant levels of stimulation, both HMG-1/2 and TFIIA were required in the presence of activator (lanes 4–6). Thus TFIIA is essential for stimulation of transcription by Gal4–VP16 in the presence of HMG-1/2.

The potential importance of TFIIB binding in activation has been described (Lin and Green 1991; Choy and Green 1993). However, some experiments suggest that at least part of the activation process precedes the interaction of TFIIB with the template-bound TFIID complex (White et al. 1992). If the function of activators and coactivators were to increase a rate-limiting TFIIB-binding step during initiation, then increasing the concentration of TFIIB should reduce the level of stimulated versus basal transcription. The concentration of TFIIB was titrated over three orders of magnitude (Fig. 5B). High levels of TFIIB did not obviate the need for activator or HMG-1/2 to attain significant levels of stimulation. As the concentration of TFIIB increased, transcription from the ML-3G template in the absence of HMG-1/2 saturated at lower concentrations of TFIIB than in the presence of HMG-1/2. This increase in the degree of stimulation with increased TFIIB concentration was also observed by Choy and Green (1993) in a TFIIB-depleted nuclear extract and suggests that activation of transcription is not equivalent to an increased affinity of the initiation complex for TFIIB.

Staged reactions on immobilized templates

The step in the assembly of the initiation complex affected by activation can be partially characterized by an immobilized template system (Lin and Green 1991). By tethering the templates to magnetic beads, distinct complexes formed on the template can be purified from unbound or loosely associated factors and tested for transcriptional activity with the subsequent addition of other factors and nucleotides. Template DNA was end-labeled with a single biotinylated deoxynucleotide and then bound to streptavidin covalently linked to paramagnetic beads. Typically, two incubations are done. The first incubation, the "pre-inc," allows the formation of a distinct preinitiation complex on the promoter. The complexes formed in the pre-inc are then washed to remove uncommitted factors. In the second incubation, the "chase," the remaining transcription factors are added along with nucleotides. Thus the effect of stimulation on the activity of basal factors in the formation of stable preinitiation complexes can be determined by preincubation of different combinations of factors.

In the presence of both activator and HMG-1/2, stimulation of transcription resulted in an increased formation of active preinitiation complex (Fig. 6A). In lanes 1 and 2, all of the GTFs were preincubated with and without Gal4–VP16 (G) respectively, washed, and chased with nucleotide triphosphate under transcription conditions. Gal4–VP16 had no effect on the formation of initiation complexes until HMG-1/2 was added during the pre-inc (cf. lanes 2 and 4). In Figure 6B, the basal factors and HMG-1/2 were preincubated in the presence and absence of the activator MLTF. As was observed with Gal4–VP16, the amount of active preinitiation complex was increased only if the activator was present in the pre-inc. Additionally, lanes 3 and 4 show that the addition of ATP during the pre-inc did not alter the amount of stimulation. If HMG-1/2 was added both in the pre-inc and afterwards in the chase, no change in the level of stimulation was observed. This strongly argues that the positive effect of HMG-1/2 does not require ATP hydrolysis and acts during preinitiation and not at a postinitiation step.

Activation occurs at the TFIID–TFIIA complex stage

To determine the first step of initiation that is altered by

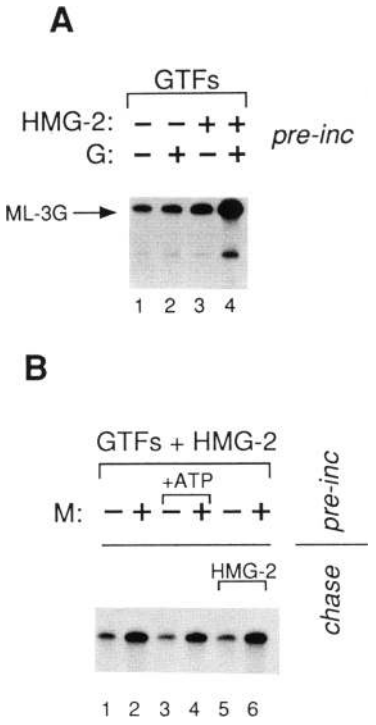

Figure 6. Preinitiation complex formation is increased in the presence of activator and HMG-2. (*A*) Linear MLP template containing the 390 nucleotide G-less cassette with three Gal4-binding sites fused upstream was biotinylated at one end and bound to magnetic streptavidin beads. The general transcription factors were incubated with template bound beads alone (lane *1*), with Gal4–VP16 (G) (lane *2*), with pHMG-2 (lane *3*), or with both Gal4–VP16 (G) and pHMG-2 (lane *4*), for 60 min at 30°C. Complexes formed on the template beads were washed, and nucleotides were added under transcription conditions. (*B*) Template beads of the MLP with five MLTF-binding sites fused upstream of the 390-nucleotide G-less cassette were prepared as described above. General transcription factors and pHMG-2 were incubated in the absence and presence of saturating amounts of purified MLTF (M) as described above. ATP was added in this preincubation to 0.1 mM in lanes *3* and *4*. Complexes formed were washed, and nucleotides were added under transcription conditions in the chase step. Additionally, pHMG-2 was added again in the *chase*, in lanes *5* and *6*.

activation, activator and HMG-1/2 were added together at distinct steps in the assembly of the initiation complex (Fig. 7). Activation was observed when all factors, including activator and HMG-1/2, were added together in the chase but was not observed if the activator and HMG-1/2 were preincubated with template (cf. lanes 1 and 2). This indicates that HMG-1/2 is probably not simply stabilizing the binding of activator on the template. When TFIID was preincubated on the template with HMG-1/2 and Gal4–VP16 (lane 4) or committed alone and then chased with both (lane 3), no activation was observed. This indicates both that TFIID alone is not recruited to the template during activation and template-committed TFIID does not subsequently support activation. However, when TFIIA and TFIID were preincubated in the presence of activator and HMG-1/2, stimulation was observed (cf. lanes 5 and 6). These results are consistent with the previous finding that TFIIA was essential for activation (Fig. 5A).

Lin and Green (1991) have reported that TFIIB associates more stably with TFIID in the presence of activators. Furthermore, they have suggested that the direct interaction of TFIIB with activator promotes the association of this protein with TFIID (Choy and Green 1993). As shown by a comparison of lanes 7 and 8 (and data not shown), preincubation of the activator, HMG-1/2, and TFIID did not increase the stable association of TFIIB significantly. However, consistent with the phenomenology of the Lin and Green (1991) result, preincubation of TFIIB with TFIID and TFIIA under activating conditions resulted in a more stable association of TFIIB (cf. lanes 9 and 10). This enhanced association clearly occurs either after or simultaneously with the formation of the activated TFIID–TFIIA complex and may reflect a difference in the conformation of the activated versus basal complexes. These data suggest that retention of TFIIB on the TFIID complex is not the first step in activation but that activation precedes this step, at the stage of interaction of activator, coactivator, TFIID, TFIIA, and template.

The electrophoretic mobility shift assay (EMSA) was used to investigate the stability of TFIID–TFIIA complexes after activation by activator and HMG-2. Interestingly, no quantitative differences were observed in the formation of the TFIID–TFIIA complex under activation conditions in the presence of HMG-2 and Gal4–VP16 (Fig. 8). Holo-TFIID and radiolabeled MLP with three Gal4 sites fused upstream were coincubated with combinations of TFIIA, HMG-2, and Gal4–VP16 and then subjected to EMSA on agarose gels. Essentially, no TFIID complex was detected in the absence of TFIIA even in the presence of activator and HMG-2 (lanes 1–4). In the presence of TFIIA, a stable TFIID–TFIIA complex formed. This complex was slightly altered in mobility

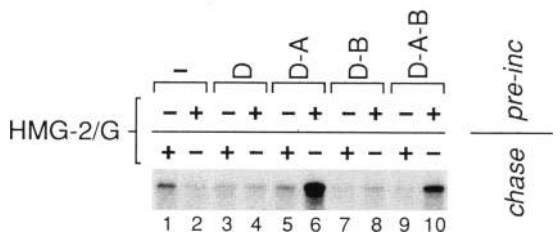

Figure 7. Activation occurs at the TFIID–TFIIA complex. Linear MLP template with three Gal4-binding sites was biotinylated and bound to magnetic beads as described (Materials and methods, Fig. 6 legend). GTFs, including homogeneously purified human TFIIA, were used to form the preinitiation complexes: TFIID (lanes *3,4*), TFIID–TFIIA (lane *5,6*), TFIID–TFIIB (lanes *7,8*) and TFIID–TFIIA–TFIIB (lanes *9,10*). Gal4–VP16 (G) and pHMG-2 were incubated with the particular factors in the *pre-inc* (even-numbered lanes) or added after complex formation in the *chase*, along with the remaining general factors and nucleotides (odd-numbered lanes).

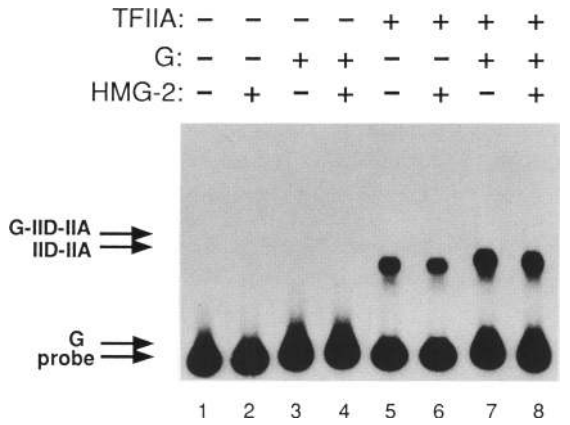

Figure 8. Formation of the TFIID–TFIIA complex. Radiolabeled MLP probe was incubated with TFIID and various combinations of homogeneously purified TFIIA, rHMG-2, and Gal4–VP16 under conditions consistent with activated transcription and analyzed by electrophoresis in 1.6% agarose–TBE gels (Lieberman and Berk 1994).

when coincubated with Gal4–VP16; however, no significant change was observed in the TFIID–TFIIA complex if HMG-2 was included with Gal4–VP16 (lanes 5–8). These last conditions generated activation in the previous transcription assays. These results suggest that during the activation process no quantitative change occurs in the formation of the TFIID–TFIIA complex.

The state of the TFIID–TFIIA complex on the promoter under basal and activating conditions was also examined using DNase I footprint analysis. No significant changes in either the extent of the footprint or its strength were observed when HMG-2 and activator were coincubated with TFIID and TFIIA on the template (Figure 9). As expected, the TBP–TFIIA complex protected the TATA region from −35 to −20 (lane 3). Both the TBP–TFIIA and the TFIID–TFIIA complexes stabilized Gal4–VP16 binding, increasing the strength of the footprint over the Gal4-binding sites. HMG-2 did not alter the TBP–TFIIA footprint (cf. lanes 3 and 5). As has been reported (Sawadogo and Roeder 1985; Lieberman and Berk 1994), the TFIID–TFIIA complex gave an extended footprint of protected and hypersensitive sites downstream of the TATA box from −35 to +40 (cf. lanes 3 and 4 with 7 and 8). However, no alteration of the footprint was observed when HMG-2 was added, either alone or with activator, in the TFIID–TFIIA-binding reaction (cf. lanes 7 and 8 with 9 and 10). Thus, under activation conditions, the presence of activator and HMG-2 did not alter the footprint of the TFIID–TFIIA complex on the promoter.

Discussion

We have purified a factor important for activation and identified it as HMG-2. Interestingly, the activity of HMG-2 is specific for some promoters. Activation of transcription requires the presence of TFIIA, and the early initiation complex composed of TFIID and TFIIA is modified by interaction with activator and HMG-1/2. Similar to activation in total extracts and in partially fractionated systems, stimulation of transcription in this system depends on the presence of TAFs in the holo-TFIID complex (Dynlacht et al. 1991; Zhou et al. 1992) and results in a change in the nature of the interaction of TFIIB with the activated preinitiation complex. We propose that this activation step involves the induction of a structure in the TFIID–TFIIA–promoter complex that facilitates initiation.

HMG-1 and HMG-2 function interchangeably in reactions containing the MLP. The HMG box from the B-subdomain of HMG-1 functions indistinguishably from the full-length protein. This subdomain consists of 80 amino acids folded into three α-helices arranged roughly into an L-shape (Read et al. 1993; Weir et al. 1993). The HMG box, as well as HMG-1 and HMG-2, binds DNA in the minor groove and bends the helix toward the major groove (for review, see Grosschedl et al.

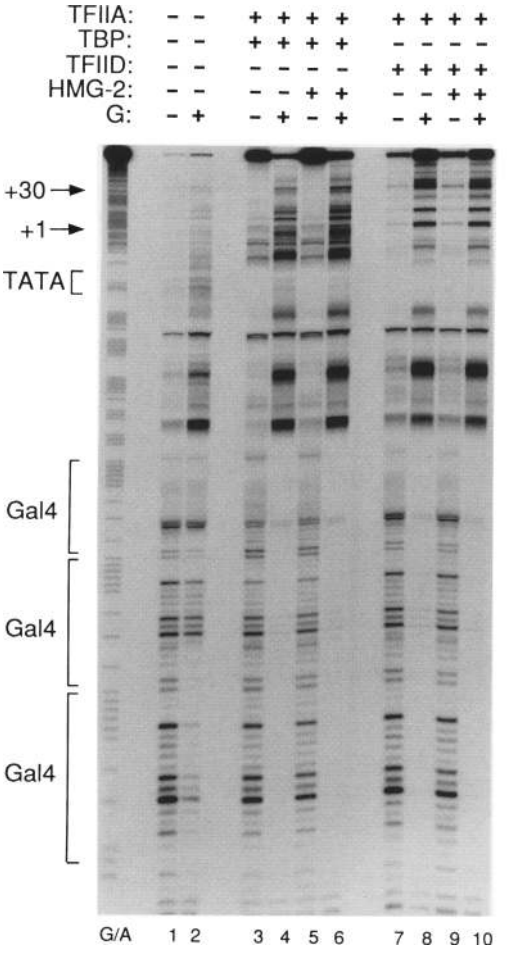

Figure 9. Footprint of the TFIID–TFIIA complex. The DNase I footprint pattern of TBP–TFIIA and TFIID–TFIIA complexes were analyzed with various combinations of pHMG-2 and Gal4–VP16. Binding reactions were identical to those used in Fig. 8.

1994). Because the HMG box subdomain alone enables activation in the system, it is likely that these proteins function by stabilizing conformations of DNA bound to the basal factors and activator. These critical conformations could involve looping of the activator to the basal complex or altering of the local topology of the DNA around the basal factors (Giese et al. 1992; Paull et al. 1993; Basett-Jones et al. 1994).

The HMG-1/2 proteins enhanced activation dramatically in reactions containing the MLP or the HSP but suppressed transcription in reactions containing the IgH μ-promoter. Maximal activation was observed at a HMG-2/template ratio of ~100:1, an absolute concentration of HMG-2 of 150 nM. On the basis of the reported affinity of HMG-1/2 for double-stranded DNA (Pil and Lippard 1992), this ratio represents only a few molecules of bound HMG-1/2 per template DNA. The template specificity of HMG-1/2 must reflect a difference in the sequence of these promoters and, consequently, a difference in the nature of their interactions with protein factors. For example, such specificity could be attributable to a sequence preference for the binding of the HMG-box to the MLP or HSP templates. However, HMG-1/2 binds DNA with little or no direct sequence specificity other than binding to irregular or bent structures (for review, see Bustin et al. 1990; Grosschedl et al. 1994 and references therein). More likely, the template specificity reflects the dependence of a critical activation complex on the enhanced stability conferred by the HMG protein. For example, a hypothetical activated conformation of the TFIID–TFIIA complex could be dependent on the topology of the promoter stabilized by HMG-1/2. We favor this latter possibility, as the IgH μ-promoter was not activated at any concentration of HMG-2 and its basal activity was repressed by low concentrations of HMG-1/2.

Activation was first detected at the stage of the interaction of TFIID, TFIIA, HMG-2, and activator in the presence of template. This activated complex is distinguishable by a decrease in the rate of dissociation of TFIIB and a more efficient assembly of a transcriptionally active polymerase complex. However, activation is not attributable to a limitation in the availability of TFIIB for binding, as the difference in activated versus basal activity could not be reduced by addition of high concentrations of TFIIB. Both results are consistent with the proposal that the conformation of the activated TFIID–TFIIA–HMG-2 complex is different than that of the equivalent basal complex. The former is conjectured to interact with TFIIB and probably other basal factors in a different fashion such that the rate of initiation is increased. The observations in these defined reactions are remarkably consistent with previous work, in which partially purified fractions were used, where TFIIB was bound more stably to the activated template than the control promoter, but high concentrations of TFIIB did not decrease the fold activation (White et al. 1992; Choy and Green 1993).

The dependence of activation in the presence of HMG-1/2 on TFIIA agrees with previous suggestions that the TFIID–TFIIA binding step is important for stimulation (Wang et al. 1992; Ma et al. 1993) and with results demonstrating the importance of TFIIA in stimulation in various less well-defined in vitro systems (Ozer et al. 1994; Sun et al. 1994; Yokomori et al. 1994). Recently, the viral activator Zta was shown to increase the rate of formation of a TFIID–TFIIA complex (Lieberman and Berk 1994). However, this enhanced rate of formation of the TFIID–TFIIA complex did not correlate directly with enhanced transcription. Thus, it was not possible to equate formation of the TFIID–TFIIA–Zta complex with the activation event. The kinetic analysis of initiation complex formation has also shown that in the presence of partially fractionated TFIID, TFIIA, and activator, the lag before open complex formation was shortened (Wang et al. 1992). However, in the present study, it is unlikely that an acceleration in the rate of formation of the TFIID–TFIIA–template complex is the critical aspect in activation, as prolonged incubations did not suppress the degree of activation (data not shown). Furthermore, activation at the TFIID–TFIIA stage was not manifested by an increase in complex formation as assayed either by footprint analysis or resolution of complexes by gel electrophoresis.

In the defined reconstituted reaction, association of TFIID and template in the absence of TFIIA rendered this complex refractory to subsequent activation but still able to support the basal reaction. The refractory nature of the template-committed TFIID suggests that the conformation of TFIID–promoter complexes can vary. This observation is similar to previous results where template-committed TFIID complexes were unable to respond to the addition of an activator (Wang et al. 1992). Those complexes formed in the absence of activator, which were referred to as "inactive" by Wang and coworkers, may be locked in a conformation that only supports basal levels of transcription.

Consistent with all of the above results, the activation event probably involves a conformational change in the TFIID–TFIIA complex, possibly involving an alternate arrangement of the TAFs on the template. The activation event may expose multiple or altered binding sites for TFIIB that in turn stabilize a specific conformation of TFIIB. This conformation of TFIIB could more efficiently recruit the other proximal basal factors for initiation. Such a conformational change in TFIIB has been postulated (Choy and Green 1993; Roberts and Green 1994), and mutants in TFIIB exist that support basal but not stimulated transcription (Roberts et al. 1993). It is also likely that the activated TFIID–TFIIA–template conformation preferentially interacts with basal factors beyond TFIIB in initiation. A Pol II holoenzyme complex, such as that defined in yeast (Koleske and Young 1994), could be recruited through multiple interactions with the activated TFIID–TFIIA complex.

In this system HMG-1/2 allows the basal reaction to respond to activators, although it is probably acting by a different mechanism than the factors defined previously as coactivators. The soluble, non-TAF coactivators described to date, although not essential for the basal re-

action, can modulate this reaction in the absence of activators, possibly altering the TFIID–TFIIA interaction. The defined transcription reaction described here, which mediates activation with HMG-1/2, does not contain any of the known soluble coactivators. The USA component PC4 has been shown to interact with the basal factors and with the activation domains of activators and thus is probably acting as an "adapter" molecule, physically linking the activator and the basal machine. HMG-1 does not interact with activators (Ge and Roeder 1994b; B.M. Shykind et al., unpubl.), but has been reported to form a complex with TBP on the promoter (Ge and Roeder 1994b). Interestingly, HMG-1/2 has been reported to repress both the basal and stimulated reactions in vitro (Ge and Roeder 1994b; Stelzer et al. 1994). We have found that the presence of either topoisomerase I or the USA component PC4 alters reactions such that HMG-1/2 represses transcription upon titration (B. Shykind, J. Kim, and P. Sharp, in prep.). Topoisomerase I/PC3 antagonizes the formation of the TBP–TFIIA complex on the promoter (Merino et al. 1993) and thus could alter the nature of the interaction of activators with the TFIID–TFIIA complex. The enzymatic activity of topoisomerase I is dispensable for its repression of transcription (Merino et al. 1993).

HMG-1 and HMG-2 are the founding members of a family of DNA-binding proteins involved in transcription, including LEF-1 (Giese et al. 1992), SRY (Ferrari et al. 1992), hUBF (Jantzen et al. 1992), and DSP-1 (Lehming et al. 1994), all of which contain one or more HMG boxes (for review, see Grosschedl et al. 1994). The ability of the HMG box-containing factors to modulate DNA conformation has led to the description of this family as "architectural" transcription factors (Wolffe 1994). That HMG-1/2 potentiates stimulation in the presence of purified basal factors and activator suggests that the activation step does not require components beyond the basal machinery, activator, and an architectural factor. We propose that this activation step involves the stabilization by the HMG-1/2 proteins of a specific conformation of the TFIID–TFIIA–activator complex that facilitates enhanced rates of initiation.

Materials and methods

Purification of Co-B

The preparation of crude MLTF fraction, HBrBC, which contained HMG-2, was described in detail previously (Chodosh 1988) and is summarized briefly here. Nuclear extract was made from homogenized calf brain with minor modifications to the method of Dignam et al. (1983). The extract was equilibrated in buffer A [20 mM HEPES–NaOH (pH 7.9), 20% glycerol, 1 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride (PMSF)] plus 100 mM KCl, and 0.03% NP-40 and heated at 80°C for 35 min with continuous stirring. After cooling to room temperature and centrifugation, the supernatant was loaded onto a DEAE–Sepharose column equilibrated in buffer A, 100 mM KCl, and 0.03% NP-40. The column was washed sequentially with three column volumes of buffer A plus 100 mM KCl, three column volumes of buffer A plus 250 mM KCl, and three column volumes of buffer A plus 500 mM KCl. MLTF activity, assayed by EMSA, was found in the 250 mM step (HBrB). Heparin–Sepharose (Pharmacia) was prepared according to the manufacturer's instructions and was equilibrated in buffer A plus 100 mM KCl. After loading the HBrB, the column was washed sequentially with three column volumes of buffer A plus 100 mM KCl, three column volumes of buffer A plus 250 mM KCl, and three column volumes of buffer A plus 500 mM KCl. MLTF was found in the 500 mM KCl step (HBrBC).

Phosphocellulose (Whatman P11) matrix (1.3 ml) was prepared according to the manufacturer's instructions and equilibrated with buffer A plus 200 mM KCl. HBrBC (7 mg in 7.5 ml) was batch absorbed and washed sequentially with four column volumes of buffer A plus 200 mM KCl, four column volumes of buffer A plus 350 mM KCl, four column volumes of buffer A plus 600 mM KCl, and four column volumes of buffer A plus 1 M KCl. The MLTF was found in the supernatant of binding and 350 mM KCl step. The Co-B activity, found in the 600 mM KCl step, was dialyzed to buffer A plus 100 mM KCl and loaded onto a Mono-Q HR 5/5 column (FPLC, Pharmacia). The bound material was eluted with a 12-ml linear gradient of 100–400 mM KCl in buffer A. The activity was eluted near 300 mM KCl. The active fractions were dialyzed to buffer A plus 1.2 M ammonium sulfate and loaded onto a phenyl-Sepharose HR 5/5 column (FPLC, Pharmacia). The loaded material was eluted with a 12-ml linear gradient of 1.2–0 M ammonium sulfate in buffer A. The activity, found in the flowthrough, was dialyzed to buffer A plus 100 mM KCl and loaded onto a Mono-S HR 5/5 column (FPLC, Pharmacia). From a 12-ml linear gradient of 100 mM to 1 M, the peak of the activity was found near 200 mM KCl. Further processing of the purified material for renaturation and amino acid sequencing was done using a C4 reverse-phase column (HPLC system, HP model 1090M, part 214TP52).

Renaturation of HPLC fractions

Each of the fractions from the C4 column was brought to 0.1 M HEPES (pH 7.9), frozen, and then lyophilized. To each fraction, 15 μl of denaturation buffer [6 M guanidine-HCl, 50 mM Tris (pH 7.9), 0.1 mM EDTA, 10% glycerol, 50 mM NaCl, 2 mM DTT] was added to solubilize the dried material thoroughly. After incubating at room temperature for 15 min, 105 μl of dilution buffer [50 mM Tris (pH 7.9), 5% glycerol, 150 mM KCl, 0.1 mg/ml of BSA, 0.1 mM EDTA, 2 mM DTT] was added. Fractions were dialyzed overnight to buffer A plus 100 mM KCl at 4°C.

Amino acid sequencing of Co-B

Amino-terminal sequencing of the sole polypeptide in the active renatured fraction (fraction C) was carried out on an Applied Biosystems model 477A protein sequencer with on-line model 120 PTH amino acid analyzer. The amino-terminal 21 amino acids revealed a sequence that matched that of the HMG-2 protein from several species, including bovine HMG-2 (see Results).

Expression of HMG-2 in Escherichia coli

A pair of primers bearing the amino- and carboxy-terminal ends of HMG-2-coding sequence flanked by *Bam*HI restriction sites were generated (amino terminus, 5′-dGCACGGATCCAGGTAAAGGAGACCCCAACAAGCCG-3′; carboxyl terminus, 5′-dGCACGGATCCTCATTATTCTTCATCTTCATCCTCTTCCT-3′). These oligonucleotides were used to generate a PCR product containing the complete coding sequence of human HMG-2 using a cDNA clone (a gift of M. Seidman of Otsuka Pharmaceuticals; Majumdar et al. 1991) as the template. The

PCR product was digested with *Bam*HI and cloned into pET-15b (Novagene). *Escherichia coli* strain BL21DEpLysS (Novagen) containing pHMG-2 was grown in LB medium supplemented with 150 μg/ml of ampicillin. Upon reaching an OD_{600} of 0.6, HMG-2 was induced with 1 mM IPTG for 3 hr. The harvested cells were resuspended in buffer L [20 mM Tris (pH 7.9), 200 mM NaCl, 20 mM β-mercaptoethanol, 1 mM PMSF; 20 ml/liter of culture] and lysed by sonication. Induction and subsequent purification of HMG-2 were followed by Western assay with polyclonal antibody against rat HMG-1 (gift of S. Lippard). The supernatant of the lysis was sequentially precipitated with 45% and 75% ammonium sulfate. HMG-2, found mostly in the 75% ammonium sulfate precipitate, was dialyzed to buffer N [20 mM HEPES (pH 7.9), 20% glycerol, 7 mM β-mercaptoethanol, 1 mM PMSF, 0.1% NP-40], 5 mM imidazole, and 1 M KCl. This was batch absorbed onto 1 ml of Ni–NTA resin (Qiagen). The matrix was poured into a small column and washed with 10 column volumes of buffer N plus 100 mM KCl. HMG-2 was subsequently eluted with buffer N, 250 mM imidazole, and 100 mM KCl. The peak of activity was dialyzed to buffer A plus 500 mM KCl and loaded onto a Superdex 200 16/60 column (FPLC, Pharmacia). The active fractions were dialyzed to buffer A plus 100 mM KCl and loaded onto a Mono-Q HR 10/10 column (FPLC, Pharmacia). A linear gradient elution from 100 mM to 1 M KCl resulted in a homogeneous recombinant HMG-2 preparation.

In vitro transcription

Preparation of the recombinant transcription factors, TBP, IIB, IIE, and IIF, has been described (Parvin et al. 1994). Purification of holo-TFIID was done essentially as described (Zhou et al. 1992). RNA Pol II was the highly purified preparation from a CHO cell line (Carthew et al. 1988). The source of TFIIA was either A/J fraction (Parvin et al. 1994) or a homogeneous preparation of TFIIA. The latter was prepared following the steps of a previously published purification through the single-stranded DNA agarose chromatography step (Cortes et al. 1992). The active fractions were purified to homogeneity with Ni–NTA resin (Qiagen). TFIIH was purified from HeLa nuclear extract-derived fraction C1 (Parvin et al. 1992). The purification was followed by Western assay using an antibody to the p62 subunit (gift of J. Egly; Fischer et al. 1992). C1 fraction (80 mg in 50 ml) in buffer A plus 100 mM KCl was loaded onto a Mono-Q HR 10/10 column (FPLC, Pharmacia). An 80-ml linear gradient from 100 to 600 mM KCl was used to elute the TFIIH complex. The peak fractions, near 230 mM KCl, were dialyzed to 1.0 M ammonium sulfate in buffer A and loaded onto a phenyl–Sepharose HR 5/5 column (FPLC, Pharmacia). The loaded material was eluted with a linear gradient from 1.0 M to 0 M ammonium sulfate in buffer A. The activity, found near 300 mM ammonium sulfate, was dialyzed to buffer A plus 500 mM KCl and loaded onto a Superdex 200 16/60 column (FPLC, Pharmacia). TFIIH, by Western assay and transcription, was found to have an apparent size of 700 kD. Gal4–VP16 was prepared as described (Chasman et al. 1989). Rat HMG-1 and rat HMG1 boxB were kind gifts of S. Lippard (Pil and Lippard 1992). MLTF used in the transcription assay was the phosphocellulose flowthrough fraction described above.

DNA templates for transcription in vitro, Gal4(3)–MLP, and MLTF(5)–MLP, were gifts of H. Timmers (University of Utrecht, The Netherlands) and S. Harper (Massachusetts Institute of Technology, Cambridge, MA), respectively. Gal4(3)–μ and Gal4(5)–HSP were gifts of J. Parvin. The control template MLP has been described (Timmers and Sharp 1991). The conditions for the assay and typical amounts of basal transcription factors used were as described previously (Parvin et al. 1994). Where indicated, 10 ng of Gal4–VP16, 1 μl of MLTF fraction, and 100 to 500 ng of purified or cloned HMG-1/2 preparations were added.

The immobilized templates were generated by cutting the appropriate templates with *Xmn*I and *Nde*I followed by Klenow end-filling with biotin-16–dUTP (Boehringer Mannheim) and dATP (100 μM each). The gel-purified 2500-bp fragment was incubated with streptavidin-coupled M-280 Dynabeads (Dynal Inc.; 10 μl of slurry/100 ng of DNA) for 60 min. The DNA-coupled resin was washed and stored in buffer A, 100 mM KCl, and 0.1% NP-40 in the original volume.

Staged transcription assays were typically done as follows. The DNA-coupled resin (10 μl) was incubated with the pre-inc factors for 60 min at 30°C in buffer A, 100 mM KCl, 0.1% NP-40, and 5 mM $MgCl_2$ (total volume, 20 μl). The protein–DNA complexes were washed with 300 μl of the incubation buffer. The chase factors and nucleotides were added subsequently, and the reaction was brought to the transcription condition (see above). The incubation was continued for 90 min at 30°C.

EMSA and DNase I footprinting

A pair of primers were generated to produce a 260-bp PCR product from a Gal4(3)–MLP transcription template (5′-dGACCATGATTACGCCAAGCTTGCATGCCTGC-3′; 5′-CGCTACTCGAGAGGAATAATGAGGAAAGGAGAGTAG-3′). The PCR product, covering the regions −180 to +65 of the promoter region, was digested with *Hin*dIII and *Xho*I and inserted into pBSII-SK+. The probe for EMSA and DNase I footprinting was generated by cutting the plasmid with *Hin*dIII, Klenow-end filling with [^{32}P] dATP, and then cutting with *Xho*I. Protein–DNA-binding reactions were done following published protocol (Lieberman and Berk 1994) with several modifications. Binding was done in 16 μl of binding buffer [12.5 mM HEPES (pH 7.9), 12.5% glycerol, 6.25 mM EDTA, 70 mM KCl, 10.8 mM β-mercaptoethanol, 0.5 mg/ml of BSA, 5 μg/ml of poly(dGdC), and 1 ng of probe] at 30°C for 30 min. Gels for EMSA were cast with 1.6% agarose in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA) with 6.25 mM $MgCl_2$. The gel was run in 0.5× TBE with 6.25 mM $MgCl_2$ at 70 V for 5 hr and then dried on DE81 paper. For the DNase I footprinting assay, 16 μl of DNase I solution containing 1 ng of DNase I (Worthington Enzymes; DPFF grade) and 5 mM $CaCl_2$ were added to the binding reaction above. After 1 min of incubation at room temperature, 90 μl of stop solution [20 mM EDTA (pH 8.0), 1% SDS, 200 mM NaCl, 250 μg/ml of yeast tRNA] was added. The DNase I digestion was analyzed by sequencing gel electrophoresis.

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