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20. *Escherichia coli* cells harboring the pK34-1, pK34-2, or pK34-3 plasmid (*19*) were induced (*19*) and lysed by freeze-thawing in 5 volumes of 50 m*M* tris-HCl (*p*H 7.5), 1 m*M* EDTA, and 1 m*M* dithiothreitol (DTT). The assay for ubiquitin-histone conjugation was carried out in 50 μl of 50 m*M* tris-HCl (*p*H 7.5), 2 m*M* ATP, 5 m*M* $MgCl_2$, and 0.2 m*M* DTT, in the presence of 50 pmol of ^{125}I-labeled ubiquitin ($\sim 1 \times 10^5$ cpm) (*21, 22*), 0.2 μg of bovine histone H2B (Boehringer Mannheim), and 4 pmol of the purified yeast E1 enzyme (*18*). The assay for ubiquitin thiolesters was carried out as above except that histone H2B was omitted, and the reaction products were incubated for 5 min at room temperature in an SDS-containing electrophoretic sample buffer lacking reducing agents before a polyacrylamide–SDS gel electrophoresis at 4°C (*22*). Reduction of thiolesters was performed by heating the samples prior to electrophoresis at 100°C for 10 min in an SDS sample buffer containing 4*M* 2-mercaptoethanol (*18, 21*).
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33. A 3.2-kb Bgl II–Sph I fragment and a 3.3-kb Hind III–Sal I fragment from pCDC34-79 (Fig. 1) were subcloned into pGEM-1 (Promega Biotech, Madison, WI); the resulting plasmids were designated pGEM34 B/S and pGEM34 H/S, respectively.
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Physical Analysis of Transcription Preinitiation Complex Assembly on a Class II Gene Promoter

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Transcription of protein-encoding genes by human RNA polymerase II requires multiple ancillary proteins (transcription factors). Interactions between these proteins and the promoter DNA of a viral class II gene (the major late transcription unit of adenovirus) were investigated by enzymatic and chemical footprinting. The experiments indicated that the assembly of functionally active RNA polymerase II–containing transcription preinitiation complexes requires a complete set of transcription factors, and that both specific protein-DNA and protein-protein interactions are involved. This allows individual steps along the transcription reaction pathway to be tested directly, thus providing a basis for understanding basic transcription initiation mechanisms as well as the regulatory processes that act on them.

GENE ACTIVITY IN EUKARYOTES IS frequently regulated at the level of DNA transcription. Understanding the molecular mechanism behind this transcriptional control of gene expression has become one of the major goals of modern biology. Mutational analysis has revealed that the activity of protein-encoding genes is governed by several kinds of promoter elements. A basal level of transcription is usually observed when all but a small region of the promoter DNA has been deleted. This minimum or core promoter is often centered on a TATA box sequence (*1*). Two classes of cis-acting DNA elements are involved in modulating the TATA box–driven transcription. Upstream promoter elements act at short distance, while enhancer elements can be located up to several thousand base pairs from the transcription initiation site (*2*). The various components of the human class II transcription machinery are being elucidated by the biochemical dissection of crude cell-free systems (*3, 4*). This approach has revealed that specific transcription initiation by RNA polymerase II requires the coordinated action of multiple protein factors. These can be classified into two categories: general transcription factors and upstream element-binding proteins (*2*). Through both kinetic analysis and the use of various inhibitors, several steps have been defined within the transcription mechanism (*5–7*). These include: (i) a commitment of the template after binding of a subset of the transcription factors, (ii) formation of an activated state through the action of other transcription factors and RNA polymerase II, (iii) fulfillment of an energy requirement (hydrolysis of the β-γ phosphate bond of either ATP or dATP), (iv) initiation of transcription (formation of the first phosphodiester bond), and (v) transcription elongation, with some transcription components remaining committed to the template. Within these various steps, the exact roles of the different protein factors, their assembly into active transcription complexes, and the various transitions that lead to the initiation event, remain for the most part unknown.

The major late (ML) promoter of adenovirus provides a useful model system for in vitro analysis of specific transcription by human RNA polymerase II. This promoter is quite simple structurally, with only two essential DNA elements: a TATA box at position −28 and a single upstream element at position −58 (*8–11*). At least three transcription factors (designated TFIIB, TFIID,

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and TFIIE) are absolutely necessary, in addition to the RNA polymerase II itself, for a basal level of expression driven by the ML TATA box (*4*). Maximal activity of the promoter requires in addition a gene-specific transcription factor designated upstream stimulatory factor (USF) (*10*) [or MLTF, UEF (*11*)]. DNA cleavage protection methods (footprinting) have been used successfully to analyze the primary protein-DNA interactions necessary for ML transcription, namely the binding of TFIID to the TATA element (*10, 12*) and of USF to the ML upstream element (*10*). We report here that the same methods can be used to visualize directly subsequent steps along the reaction pathway.

Our in vitro reconstituted transcription system, composed of HeLa cell–derived transcription factors TFIIB, TFIID, TFIIE, USF, and RNA polymerase II (*13*), has been described (*4, 10*). For a footprinting analysis of transcription complexes, USF and TFIID were first incubated with a small amount of ^{32}P-labeled DNA containing the ML promoter under suitable conditions for complete binding (*10*). These preformed complexes were then subjected to a second incubation with the other protein fractions under transcription conditions (except for the absence of nucleotide triphosphates and the addition of a large concentration of nonspecific carrier DNA). We first investigated the transcription factor requirement for the assembly of complete preinitiation complexes (Fig. 1). The deoxyribonuclease I (DNase I) footprinting pattern observed for the USF-TFIID-ML promoter complex (lane 3) is characterized by complete cleavage protections centered over the upstream element and TATA boxes, with a series of periodic enhanced cleavages observed between positions −2 and +30 (*10*). This pattern of protection did not change when subsets of the remaining transcription factors were subsequently added (lanes 4 to 6). Only with the simultaneous addition of TFIIB, TFIIE, and RNA polymerase II was a novel DNase I footprint observed (lane 7). This footprint was characterized by a loss of the periodic pattern of DNase I–enhanced cleavages in the downstream portion of the TFIID footprint, and the appearance of a new hypersensitive site downstream at position +35. This observation concurred with the earlier studies indicating that at least one protein in each of these fractions was absolutely required for specific transcription initiation at the ML promoter (*4*). Our inability to detect interactions between the USF-TFIID-ML promoter complex and subsets of the remaining transcription factors could reflect greater instabilities and lower concentrations of these intermediates. By contrast, the whole preinitiation complex could be stabilized by multiple protein-protein and protein-DNA interactions (see below).

Both the involvement of DNA sequences deemed important by mutational studies and the requirement for all previously identified transcription factors supported the idea that the above-described novel footprint reflected the assembly of transcription preinitiation complexes onto the promoter of the ML gene. However, it was essential to determine whether a majority of the complexes formed under these conditions were fully functional, that is, responsive to known RNA polymerase II effectors (substrates, inhibitors, and cofactors). For example, transcription initiation should take place upon addition of ribonucleoside triphosphates. In the template construction used for these experiments, the ML promoter drives transcription of an artificial DNA fragment lacking G residues on the RNA-like strand (C_2AT cassette) (*4*). Thus transcription through this region only requires the three nucleotides ATP, CTP, and UTP. In the absence of the fourth nucleotide, progression of the RNA polymerase should cease at the end of the cassette. Finally, a low concentration of the specific inhibitor α-amanitin should prevent transcription initiation by RNA polymerase II.

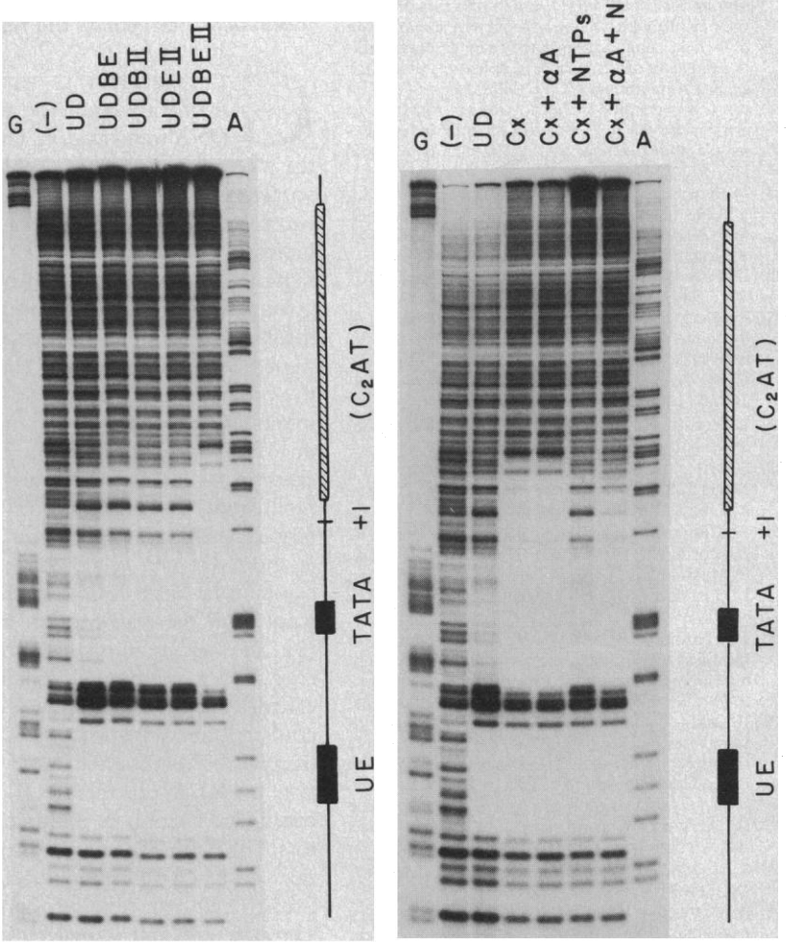

Fig. 1 (left). Transcription factor requirement for preinitiation complex assembly. Transcription complexes were assembled on the ML promoter of adenovirus [Xba I to Nar I 690-bp restriction fragment from plasmid pML(C_2AT)19Δ-127f (*10*) labeled upstream of the promoter] after a two-step incubation protocol. First, TFIID [D, 1.7 units, ω-aminooctyl agarose fraction (*23*)] and USF (U, 80 fmole; DE52 fraction) were incubated for 60 min at 30°C with 2 ng of the probe and 20 ng of carrier poly[d(GC)] in a 25-μl reaction. Subsequent incubation with the other transcription factors TFIIB (B, 2.3 units, single-stranded DNA agarose fraction), TFIIE (E, 2.3 units, Bio-Gel A-1.5m fraction), and RNA polymerase II (II, 20 units, phosphocellulose fraction) was carried out at 30°C for 30 min in a 50-μl final volume under transcription conditions (*4*) except for the absence of nucleoside triphosphatases and the presence of 800 ng of carrier poly[d(GC)]. These complexes were then analyzed by DNase I footprinting as described (*10, 16*). The products of adenosine- (A) and guanosine-specific (G) chemical-sequencing reactions (*24*) were used as markers. A schematic representation of the DNA fragment is shown at right, indicating the location of the USF-binding element (UE), TATA box, initiation site (+1), and transcription cassette (C_2AT). **Fig. 2 (right).** Footprinting analysis of transcription complexes in the presence of α-amanitin and nucleotides. Transcription preinitiation complexes (Cx) were assembled as described in Fig. 1. Where indicated, α-amanitin (αA, 2 μg/ml) or nucleotides (NTP's, 0.3 m*M* each of ATP, CTP, UTP; 0.2 m*M* 3′-*O*-methyl GTP) were present during the last 10 min of the second incubation. Control reactions contained either no transcription factors (−) or TFIID and USF only (UD).

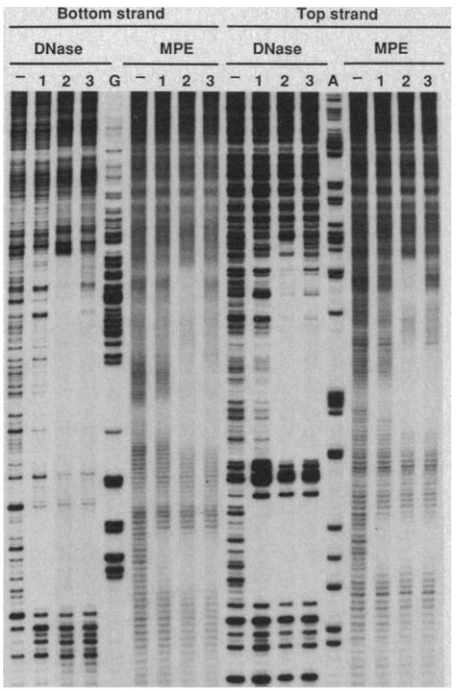

Fig. 3. High-resolution footprinting analysis of transcription complexes. Reactions containing either no protein (lane –), USF and TFIID (lane 1), all transcription factors and RNA polymerase II (lane 2), or all transcription factors and RNA polymerase II together with α-amanitin and nucleotides (lane 3) were performed with DNase I or MPE (*10, 16*) as indicated. Either 3′ or 5′ end-labeling of the DNA fragment was used to reveal protein interactions with either the top or bottom strand of the ML promoter, respectively. Cleavage products were resolved through electrophoresis on a 40-cm, 8% acrylamide-sequencing gel along with adenosine-specific (A) and guanosine-specific (G) chemical-sequencing ladders as markers.

When nucleotides were added to transcription complexes, the original large footprint surrounding the TATA box and transcription initiation site disappeared and was replaced by a cleavage pattern identical to the initial TFIID footprint (Fig. 2, lanes 3 and 6). Furthermore, a new region of DNase I protection appeared at the end of the cassette (lane 6). Both these changes were prevented when α-amanitin was added together with the nucleotides (lane 7). We interpret these results as follows. In the presence of nucleotides, RNA polymerase II initiates transcription and clears the promoter, leaving behind a subset of the transcription factors (minimally USF and TFIID). The new footprint at the end of the C_2AT cassette reflects RNA polymerase arrested in the elongation process by the absence of the next required nucleotide, GTP. From this we conclude that a sizable majority of the labeled templates had been assembled into active transcription complexes.

α-Amanitin alone had no effect on the footprint afforded by the preinitiation complex (Fig. 2, lane 5). However, a slight change was observed in the presence of nucleotides and α-amanitin, evidenced primarily by a loss of the hypersensitive cleavage site at the 3′ boundary (compare lanes 4, 5, and 7). Further analysis revealed that this same alteration took place on addition of only ATP or dATP (*14*). Therefore this transition may correspond to the previously described energy-dependent step in transcription initiation by RNA polymerase II (*6*).

DNase I footprinting is a convenient method for the initial detection of site-specific protein-DNA complexes. However, more detailed information can often be obtained with other cleaving agents like methidiumpropyl-EDTA · Fe(II) (MPE) (*15*). With its small molecular mass, nonspecific cleavage mechanism, and moderately strong association with DNA, MPE has proved useful for delineating regions of strong protein-DNA interaction (*10, 16*). In order to further characterize the transitions between the various transcription complexes, we used both DNase I and MPE footprinting in conjunction with high-resolution gel electrophoresis to investigate the precise location of protein-DNA contacts on each strand of the ML promoter (Figs. 3 and 4). The footprints afforded by TFIID and USF alone (Fig. 3, lane 1; Fig. 4, row 1) were essentially identical to those previously described, with MPE protections outlining the sequence-specific interactions of TFIID with the TATA box and of USF with the ML upstream element. Formation of the complete transcription preinitiation complex was evidenced by the appearance of new protected regions downstream of position −40 (Fig. 3, lane 2; Fig. 4, row 2). Within the large region of DNase I protection (positions −41 to +33), the MPE footprints delineated two discrete areas of strong protein-DNA interactions. The first one included the TATA box, yet was broader than the initial TFIID footprint. A second region of MPE protection was located between positions +1 and +24. Since these latter sequences lie mostly outside of the ML promoter and within the artificial C_2AT

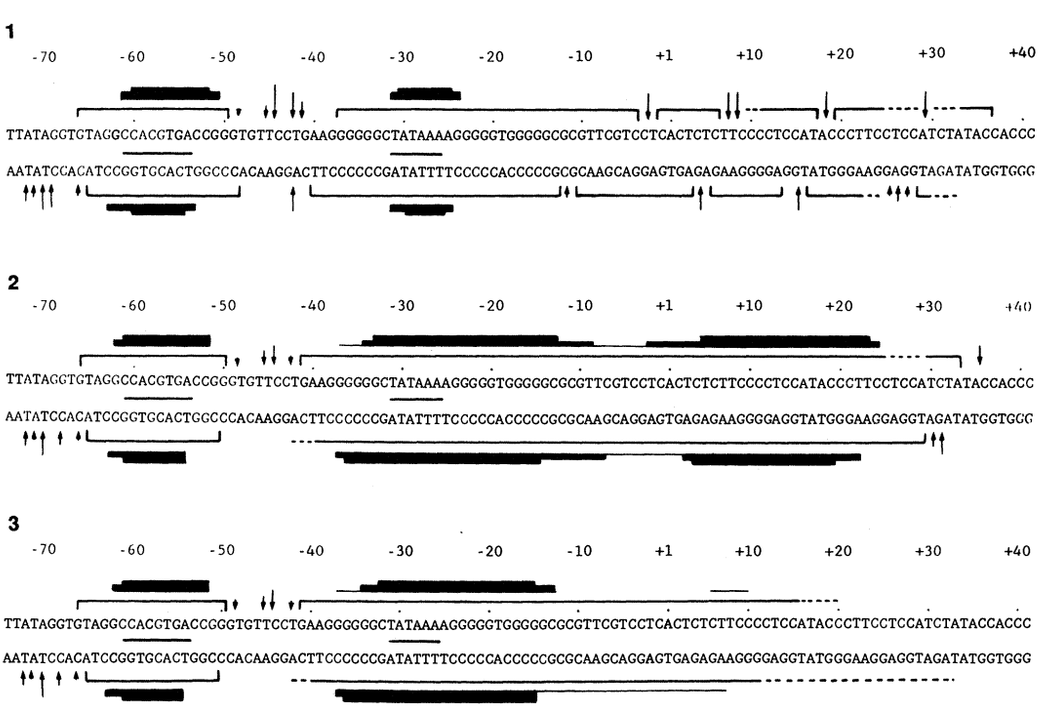

Fig. 4. Summary representation of the interactions between transcription factors and the ML promoter. Cleavage protections derived from the data in Fig. 3 are schematically represented with the following conventions: DNase I footprints are indicated by brackets; sites of enhanced DNase I cleavage, by arrows whose length is proportional to the degree of enhancement. MPE footprints are shown as histograms. (Row 1) USF and TFIID; (row 2) transcription preinitiation complex; (row 3) transcription preinitiation complex in the presence of nucleotides and α-amanitin.

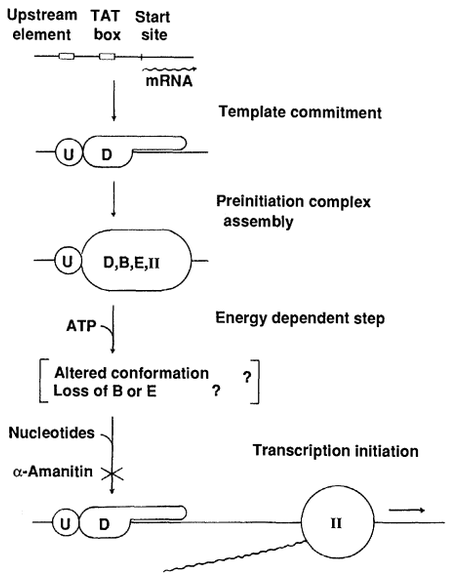

Fig. 5. Model for the mechanism of specific transcription initiation by RNA polymerase II.

cassette, protein-DNA contacts in this region are presumably DNA sequence-independent. In the presence of nucleotides and α-amanitin, the protein-DNA interactions within the transcription preinitiation complexes were significantly altered (Fig. 3, lanes 3; Fig. 4, row 3). This transition was detected by DNase I footprinting as a retreat at the downstream edge of the protected region, together with a loss of the distinctive enhanced cleavage at its 3′ border. The concomitant change in the MPE cleavage protection was most noticeable, with a disappearance of the downstream footprint and a somewhat puzzling alteration of the cleavage pattern around the initiation site.

Our experiments demonstrate that physical analyses, such as DNA cleavage-protection methods, can be used to complement functional assays in dissecting the mechanism of specific transcription initiation by eukaryotic RNA polymerase II. In view of these latest results, the model illustrated schematically in Fig. 5 summarizes our current understanding for transcription of the ML gene by human RNA polymerase II. Sequence specificity of transcription initiation is governed by the initial binding of TFIID and USF to the TATA box and upstream element, respectively. This ternary complex is further stabilized by a direct interaction between the two specific DNA binding proteins (*10*). The initial (rate-limiting) step observed in the functional assay most likely corresponds to the assembly of this primary complex which results in commitment of the promoter to the transcription process (*17*). A subsequent step leads to the formation of the whole transcription preinitiation complex and requires the other transcription factors (TFIIB and TFIIE) as well as the RNA polymerase itself. Formation of this complex results in the appearance of specific, though sequence-independent, protein-DNA contacts. Thus, these contacts must be controlled primarily by specific protein-protein interactions (for example, physical contacts between the RNA polymerase and the TFIID-USF complex). In this, the assembly of RNA polymerase II–containing preinitiation complexes presents similarities with the binding of *Acanthamoeba* RNA polymerase I and TIF factor to the cognate ribosomal gene promoter (*18*). Therefore, a general feature of eukaryotic RNA polymerases may be that promoter recognition is primarily governed by specific protein-protein interactions, rather than through sequence-specific DNA binding as is the case for prokaryotic RNA polymerases (*19*). Fulfillment of an energy-requiring step leads to a transition in the complex which could reflect the dissociation of a component or a global conformational change (*20*). This step would perhaps involve a melting of the DNA double helix, a prerequisite for transcription of double-stranded DNA templates. The energy-dependent step for eukaryotic RNA polymerase II would then be the equivalent of the closed-to-open transition for prokaryotic transcription (*19*). After transcription initiation and promoter clearance by the RNA polymerase, some remnant of the transcription complex remains associated with the ML promoter (minimally USF and TFIID) (*21*). This concurs with the earlier observation of post-transcriptional template commitment promoting reinitiation (7). However, multiple transcripts are seldom synthesized from the same promoter in our more purified reconstituted system. Thus, while the observed post-elongation complex may be necessary for template commitment, it is operationally insufficient for transcription reinitiation. Elaboration of our model requires additional studies concerning the molecular architecture of the various transcription complexes. These studies should extend our understanding of the general transcription mechanisms and provide a basis for investigating the action of regulatory factors (*22*).

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