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Mechanism of promoter selection by RNA polymerase II: Mammalian transcription factors α and $\beta\gamma$ promote entry of polymerase into the preinitiation complex

(messenger RNA synthesis/core promoter/runoff transcription)

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ABSTRACT **Productive binding of RNA polymerase II at the core region of TATA box-containing promoters is controlled by the action of the TATA factor and four additional transcription factors, designated α, $\beta\gamma$, δ, and ε, which have each been purified to near homogeneity from rat liver. This process is accomplished in three distinguishable stages. In the first stage (initial complex formation), the core promoter is packaged with the TATA factor into a binary complex that serves as the recognition site for RNA polymerase II. Here we show that, in the second stage (site selection), transcription factors α and $\beta\gamma$ act in combination to promote selective binding of RNA polymerase II to the initial complex. Several lines of evidence argue that α and $\beta\gamma$ function at this stage by a mechanism related to that utilized by bacterial σ factors. In the third stage, transcription factors δ and ε promote assembly of the functional preinitiation complex. Our evidence supports the model that δ and ε enter the preinitiation complex and direct formation of stable protein–DNA contacts that anchor the transcription apparatus to the core promoter at sequences near the cap site.**

Selective binding of RNA polymerase II at its promoter is a crucial stage in the synthesis of eukaryotic mRNA. In prokaryotes, this stage is referred to as site selection and is controlled by the action of a class of proteins known as σ factors (1). These transcription factors associate with bacterial core RNA polymerases to reconstitute a holoenzyme capable of binding selectively at promoters. The best characterized of the σ factors is the major *Escherichia coli* transcription initiation factor σ^{70}, which expedites site selection (*i*) by decreasing the affinity of *E. coli* RNA polymerase for nonpromoter sites in DNA and (*ii*) by increasing the affinity of the enzyme for its recognition site at the promoter (2, 3).

In eukaryotes, biochemical studies of partially purified transcription systems derived from HeLa cells have thus far provided a significantly different model for selective binding of RNA polymerase II at the core region of TATA box-containing promoters (reviewed in refs. 4 and 5). Whereas bacterial RNA polymerases, with their associated σ factors, bind selectively to free promoter DNA, RNA polymerase II does not. Instead, substantial evidence indicates that a general transcription factor, referred to as the TATA factor (TFIID or BTF1), first binds specifically to the TATA region of the core promoter to form an initial complex. RNA polymerase II, assisted by additional general factors, then recognizes and binds to this nucleoprotein complex to form a functional preinitiation complex, which is capable of initiating transcription rapidly when provided with ribonucleoside triphosphates.

How these additional general factors function in site selection has been the subject of much investigation. Based on kinetic and template challenge experiments, Fire *et al.* (6) and Reinberg *et al.* (7) originally proposed that RNA polymerase II enters the preinitiation complex by recognizing and binding directly to the initial complex, without the assistance of auxiliary transcription factors. According to this model, the additional general transcription factors function only after polymerase has become associated with the nucleoprotein promoter complex. Based on gel mobility-shift experiments, Buratowski *et al.* (8) recently suggested that, instead, binding of RNA polymerase II to the initial complex depends on a general transcription factor, designated TFIIB, which they proposed acts as a "bridging factor" that binds directly to the initial complex, prior to entry of RNA polymerase II into the preinitiation complex, to form a stable intermediate that functions as the recognition site for polymerase at the core promoter. Although these two models differ on what constitutes the nucleoprotein recognition site for polymerase at the core promoter, implicit in both models is the assumption that RNA polymerase II has the intrinsic ability to recognize that site, without the aid of associated σ-like transcription factors.

To explore the process of site selection by RNA polymerase II, we have used a highly purified, reconstituted transcription system derived from rat liver. In this system, productive binding of RNA polymerase II at the core region of TATA box-containing promoters requires the action of the TATA factor and four additional transcription factors designated α (9), $\beta\gamma$ (10), δ (11), and ε (12). Our previous mechanistic studies implicated $\beta\gamma$ and α, δ, or both factors in site selection (13–15). The observation that α shares structural properties with TFIIB from HeLa cells (9, 16) suggested that α, like TFIIB, might function as a bridging factor and interact stably with the initial complex to form part of the recognition site for RNA polymerase II at the core promoter. Furthermore, our finding that $\beta\gamma$ regulates nonselective binding of RNA polymerase II to free DNA and, in this respect, shares functional properties with *E. coli* σ^{70} suggested that $\beta\gamma$ might play a role in site selection (15).

We have investigated these possibilities further and report here on findings that support the model that α and $\beta\gamma$ do indeed play a crucial role in site selection by RNA polymerase II. Contrary to our expectations, however, our evidence argues that α does not interact stably with the initial complex to form part of the recognition site for polymerase. Instead, the findings presented here support the model that α and $\beta\gamma$ function together to promote entry of RNA polymerase II into the preinitiation complex by increasing the affinity of the enzyme for its nucleoprotein recognition site at the promoter.

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Abbreviations: AdML, adenovirus 2 major late; TF, transcription factor.

MATERIALS AND METHODS

Preparation of RNA Polymerase II and Transcription Factors. Transcription factors α (9) and $\beta\gamma$ (10) were purified from cytosol as described. RNA polymerase II (15) and transcription factors δ (11), ε (12), and τ (13) were purified from nuclear extracts as described. Recombinant yeast TFIID was expressed and purified as described (12) from bacterial strain N5151 containing the plasmid pASY2D (17).

Assay of Runoff Transcription. Except as indicated in the figure legends, assays were performed as described (9) with ≈100 ng of *Nde* I-digested pDN-AdML (18) or pN_4 (19), 2 ng of α (fraction V), 10 ng of $\beta\gamma$ (fraction V), 40 ng of δ (fraction VI), 20 ng of ε (fraction V), 60 ng of τ (fraction V), or 50 ng of recombinant yeast TFIID (AcA 44 fraction), and 0.003 unit of RNA polymerase II. Transcription was initiated by addition of 50 μM ATP, 50 μM UTP, 10 μM CTP, 10 μCi of [α-^{32}P]CTP, and 7 mM $MgCl_2$. After 3 min, heparin and GTP were added to 10 μg/ml and 50 μM, respectively, and reaction mixtures were incubated for 30 min.

RESULTS

The Reconstituted Liver Transcription System. In addition to RNA polymerase II and the TATA factor, synthesis of accurately initiated transcripts in the reconstituted liver system requires the action of four transcription factors designated α, $\beta\gamma$, δ, and ε, which have each been purified to near homogeneity and whose properties are summarized in Table 1. Kinetic evidence indicates that these four factors act prior to RNA synthesis to facilitate productive binding of RNA polymerase II at the core promoter; in addition, results of previous template challenge experiments argue that each factor interacts directly and stoichiometrically with intermediates on the pathway leading to assembly of the functional preinitiation complex (13, 14).

Detection of Intermediates in Selective Binding of RNA Polymerase II at the Core Promoter. An assay coupling restriction site protection with runoff transcription was used to investigate the mechanism of site selection by RNA polymerase II. In particular, we sought to determine whether transcription factors α, $\beta\gamma$, and δ, either alone or in combination, govern binding of RNA polymerase II to the initial complex. Our strategy was first to identify core promoter sequences that exhibit RNA polymerase II-dependent protection from treatment with restriction enzymes during assembly of the functional preinitiation complex and then to establish which, if any, of the liver transcription factors are required for this protection. By coupling restriction site protection with runoff transcription, this assay provides a direct and sensitive method for monitoring the interactions of RNA polymerase II and transcription factors with those promoters that will ultimately be transcribed.

The restriction site protection assay was performed as follows. Plasmid pDN-AdML (18), which contains the core region of the adenovirus major late (AdML) promoter, was used as template. As diagramed in Fig. 1*A* (*Upper*), this plasmid contains *Hin*PI (*Cfo* I) sites at −11 and +181, an *Xba* I site at +15, and a *Pst* I site at +23. As shown in Fig. 1*A* (lanes 1–4), treatment of the template with any one of these restriction enzymes, prior to assembly of the preinitiation complex, abolished synthesis of full-length runoff transcripts from the AdML promoter. On the other hand, when preinitiation complexes were preassembled by preincubation of the template with RNA polymerase II and all five transcription

Table 1. RNA polymerase II transcription factors from rat liver

Liver factor	Structure	Polypeptide composition, kDa	Native size, kDa	Ref.
α	Monomer	35	38	9
$\beta\gamma$	Heterodimer	67, 31	100*	10
δ	Multisubunit	94, 85, 68, 46 43, 40, 38, 35†	390*	11‡
ε	Heterodimer	58, 34	90*	12

*Calculated from Stokes radius and sedimentation coefficient by the method of Siegel and Monty (20), assuming a partial specific volume of 0.725 ml/g.

†It is not yet clear whether each of these polypeptides is unique; one or more may be derived from larger species by proteolysis.

‡J.W.C. and R.C.C., unpublished data.

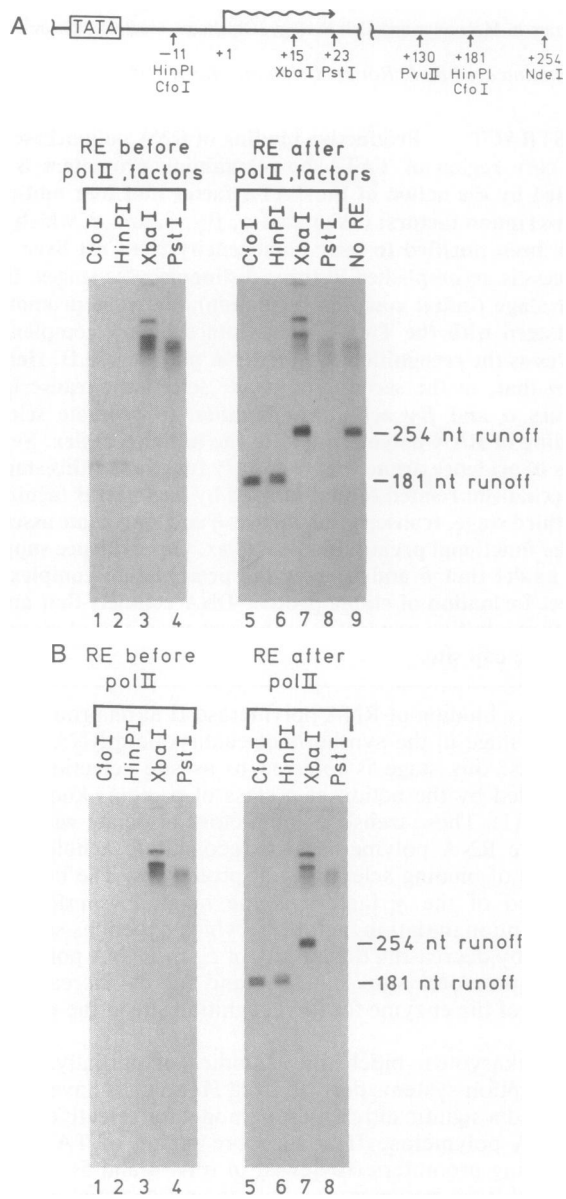

FIG. 1. Identification of core promoter sequences that exhibit RNA polymerase II-dependent protection from digestion by restriction enzymes. Runoff transcription was performed as described in *Materials and Methods* except that 7 mM $MgCl_2$ was included in all preincubations. Each reaction mixture contained 100 ng of *Nde* I-digested pDN-AdML as template. (*A*) Templates were treated for 30 min with 5 units of the indicated restriction enzyme before or after incubation with RNA polymerase II and the five transcription factors. Reaction mixtures included transcription factor τ as the TATA factor. (*B*) After preincubation with the five transcription factors templates were treated for 30 min with 5 units of the indicated restriction enzyme before or after incubation with RNA polymerase II. Reaction mixtures included recombinant yeast TFIID as the TATA factor. RE, restriction enzyme; pol II, RNA polymerase II; nt, nucleotide.

factors, prior to treatment with *Hin*PI, *Cfo* I, or *Xba* I, synthesis of correctly initiated runoff transcripts was observed (lanes 5–8), suggesting either that cleavage by *Hin*PI or *Cfo* I at −11 (but not at +181) and by *Xba* I at +15 was blocked or that a preinitiation intermediate insensitive to cleavage by these restriction enzymes was formed during the preincubation. In contrast, no full-length runoff transcripts could be synthesized from templates treated with *Pst* I, consistent with the results of previous DNase I footprinting experiments, which suggested that the RNA polymerase II preinitiation complex assembled at the AdML promoter in a HeLa cell transcription system interacts with a region extending ≈30 base pairs downstream from the cap site (21).

Protection from inhibition by *Hin*PI, *Cfo* I, and *Xba* I requires that RNA polymerase II be present in preincubations prior to treatment with restriction enzymes. As shown in Fig. 1*B*, omission of RNA polymerase II from preincubations renders both the *Hin*PI (*Cfo* I) site at −11 and the *Xba* I site at +15 sensitive to treatment with restriction enzymes; identical results were obtained when the preinitiation complex was assembled with either recombinant yeast TFIID or transcription factor τ from rat liver as the TATA factor (data not shown). Thus, these data argue that stable interaction of the transcription apparatus with core promoter sequences in the vicinity of restriction sites at positions −11 and +15 depends strongly on RNA polymerase II.

An Intermediate Dependent on Transcription Factors α and $\beta\gamma$. Order of addition experiments were performed to determine which of the liver transcription factors are essential for RNA polymerase II-dependent protection from inhibition by restriction enzymes that cut the core promoter at positions −11 and +15. Our aims here were (*i*) to discover whether all, or only a subset, of the accessory transcription factors are required for RNA polymerase II-dependent protection at these sites and (*ii*) to investigate the possibility that different subsets of factors might independently govern interactions of the transcription apparatus at the two sites. We observed that complete protection from inhibition by *Hin*PI or *Cfo* I, which cut the promoter at −11, and *Xba* I, which cuts the promoter at +15, requires RNA polymerase II, the TATA factor, and all four liver transcription factors (Figs. 2 and 3; data not shown). Thus, in addition to RNA polymerase II and the TATA factor, transcription factors α, $\beta\gamma$, δ, and ε all play a crucial role in establishing protein–DNA interactions essential for proper binding of the transcription apparatus to the core promoter.

Although protection of the *Xba* I site at +15 requires RNA polymerase II and all five transcription factors, substantial partial protection (10–30% of maximum) of the *Hin*PI (*Cfo* I) site at −11 is achieved in the presence of RNA polymerase II, the TATA factor, and transcription factors α and $\beta\gamma$; neither δ nor ε is required (Fig. 2, lanes 7 and 9). Order of addition experiments reveal that this protection depends strongly on RNA polymerase II and both transcription factors α and $\beta\gamma$ (Fig. 3). These findings are consistent with our previous observation that, in the presence of $\beta\gamma$, stable interaction of RNA polymerase II with templates containing preassembled initial complexes required α, δ, or both factors (13, 14). Moreover, these findings are consistent with the notion that transcription factors α and $\beta\gamma$, but not δ, are essential for selective binding of RNA polymerase II to the initial complex of TATA factor and promoter. The observation that, in the absence of transcription factors δ and ε, protection of the restriction site at −11 is incomplete suggests that these factors may play a role in stabilizing the binding of polymerase to the initial complex; alternatively, it is possible that, in the absence of δ and ε, the binding of polymerase to the initial complex leaves the restriction site at −11 partially accessible to the action of *Hin*PI and *Cfo* I.

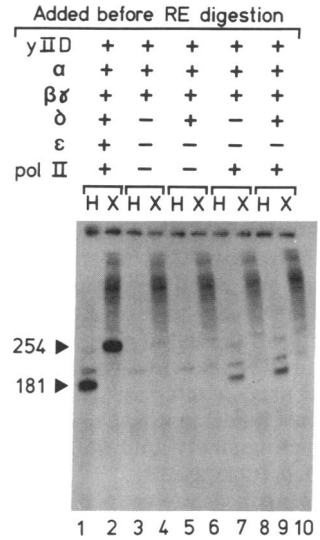

FIG. 2. An intermediate in site selection by RNA polymerase II. Runoff transcription was performed as described in *Materials and Methods* except that 7 mM $MgCl_2$ was included in all preincubations. Each reaction mixture contained 100 ng of *Nde* I-digested pDN-AdML as template and recombinant yeast TFIID as the TATA factor. The template was incubated with RNA polymerase II and factors, as indicated, before digestion for 30 min with 5 units of the indicated restriction enzyme. After digestion, the remaining components were added and the reaction was allowed to proceed. RE, restriction enzyme; pol II, RNA polymerase II; yIID, recombinant yeast TFIID; H, *Hin*PI; X, *Xba* I. Numbers on left are nucleotides.

Although these results argue that transcription factors α and $\beta\gamma$ are essential for site selection, they do not address what role these factors play in this process. It is possible, for example, that one or both factors interact stably with the initial complex to form part of the recognition site for RNA polymerase II at the core promoter, as has been suggested for the HeLa cell transcription factor TFIIB (8). The observation

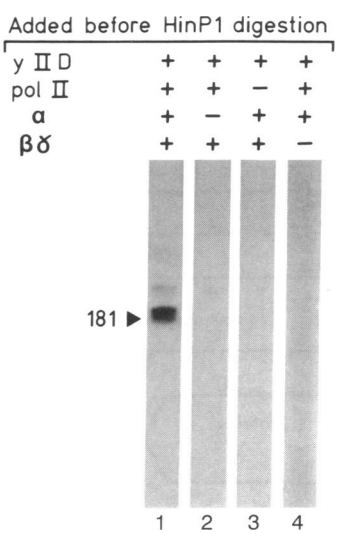

FIG. 3. Requirement for transcription factors α and $\beta\gamma$ in site selection by RNA polymerase II. Runoff transcription was performed as described in *Materials and Methods* except that 7 mM $MgCl_2$ was included in all preincubations. Each reaction mixture contained 100 ng of *Nde* I-digested pDN-AdML as template and 50 ng of recombinant yeast TFIID (AcA fraction) as the TATA factor. Templates were digested for 30 min with 5 units of *Hin*PI before or after addition of yeast TFIID, α, and $\beta\gamma$ as specified in the figure. After digestion, the remaining components were added and the reaction was allowed to proceed. pol II, RNA polymerase II; yIID, recombinant yeast TFIID. Number on left represents nucleotides.

that α shares structural properties with TFIIB suggests that α might play a similar role in site selection. On the other hand, it is also possible that one or both factors might act directly on RNA polymerase II to promote entry of the enzyme into the preinitiation complex. The observation that βγ regulates nonselective binding of RNA polymerase II to free DNA (15) suggests that βγ might function through a direct interaction with polymerase.

Stable Interaction of α with the Preinitiation Complex Requires RNA Polymerase II. Because of the similarities between α and the HeLa cell transcription factor TFIIB, we investigated the possibility that α functions in site selection by binding directly to the initial complex to reconstitute the recognition site for RNA polymerase II at the core promoter. Specifically, we used a template challenge assay (14) to determine whether, under optimal reaction conditions, α is capable of interacting stably with the initial complex in the absence of RNA polymerase II.

Template challenge assays were performed as diagramed in Fig. 4*C*. Two different templates, each containing the core region of the AdML promoter, were used. Template I was pDN-AdML (18), linearized with *Nde* I at a site 254 base pairs downstream of the cap site. Template II was pN_4 (19), linearized with *Nde* I at a site 340 base pairs downstream of the cap site. During preincubation 1, initial complexes were preassembled on template I in reaction mixture I and on template II in reaction mixture II with transcription factor τ as the TATA factor. Various combinations of α, βγ, and RNA polymerase II were then added to reaction mixtures I and II to begin preincubation 2. After this incubation, reaction mixtures I and II were combined to begin preincubation

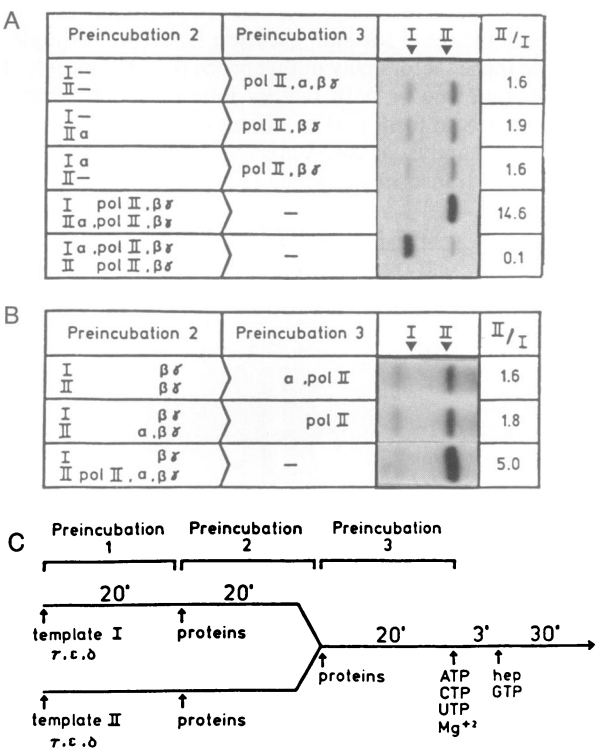

FIG. 4. Interaction of α with the preinitiation complex requires RNA polymerase II. Runoff transcription was performed as described in *Materials and Methods* and as diagramed in the figure, except that subsaturating amounts of α (0.4 ng) were included in reaction mixtures. The AdML core promoters in pN_4 and pDN-AdML are transcribed with similar efficiencies *in vitro* (22); the apparent difference in promoter strength in this experiment is not reproducible and probably results from slight differences in the concentrations of the two plasmid templates in reaction mixtures. pol II, RNA polymerase II; hep, heparin.

3. Transcription was then initiated by addition of ribonucleoside triphosphates and magnesium. If, during preincubation 2, α can interact stably with initial complexes on either template I or template II, preferential synthesis of runoff transcripts from the promoter on that template should be observed. On the other hand, if α is unable to interact with initial complexes on either template I or template II during preincubation 2, it should distribute onto both templates during preincubation 3, and roughly equivalent runoff transcription from the promoters on both templates should be observed.

As shown in Fig. 4, α does not interact stably with the initial complex in the absence of RNA polymerase II. As reported previously (14), when RNA polymerase II and the remaining liver transcription factors are present, α interacts directly and stoichiometrically with initial complexes preassembled on either template; in the absence of RNA polymerase II and βγ, however, no such stable interactions could be detected (Fig. 4*A*).

In a previous study, we observed that, in the presence of RNA polymerase II, stable association of α with a template was not dependent on βγ (14). To ensure that, in the absence of RNA polymerase II, the association of α with the template would not be stabilized by βγ, we performed the experiment shown in Fig. 4*B*. The results of this experiment indicated that, indeed, a stable interaction of α could be detected only in the presence of RNA polymerase II, even when all of the remaining transcription factors were present. Thus, α does not appear to participate in formation of a stable intermediate at the promoter prior to binding of RNA polymerase II, as would be expected if it functions as a bridging factor to reconstitute the recognition site for polymerase. In addition, consistent with previous results indicating that a stable interaction of βγ with templates containing preassembled initial complexes is strongly dependent on α (14), we observe that stable interaction of βγ with such templates depends on the presence of both RNA polymerase II and α (data not shown).

DISCUSSION

We have applied a combination of approaches to investigate the mechanism of promoter selection by mammalian RNA polymerase II in a highly purified, reconstituted transcription system derived from rat liver. Productive binding of RNA polymerase II at the core region of TATA box-containing promoters in this system requires the action of the TATA factor and four additional transcription factors designated α, βγ, δ, and ε. These five factors assemble with RNA polymerase II at the core promoter to form a functional preinitiation complex, which is capable of initiating transcription rapidly when provided with ribonucleoside triphosphates. Here we present evidence supporting a specific model for productive binding of RNA polymerase II at its promoter in the liver system (Fig. 5).

In agreement with observations made in HeLa and Drosophila K_c cell transcription systems, the first committed step in assembly of the functional preinitiation complex is binding of the TATA factor to the core promoter to form an initial complex (6, 7, 23–26). As discussed below, our evidence suggests that, in the rat liver transcription system, the initial complex serves as the recognition site for RNA polymerase II at the core promoter. In this stage, the native rat TATA factor (designated τ) is efficiently replaced by recombinant yeast TFIID (12).

After assembly of its nucleoprotein recognition site at the core promoter, RNA polymerase II, with the assistance of α and βγ, enters the preinitiation complex (stage 2, site selection). Our findings support the model that these factors promote selective binding of RNA polymerase II to the initial

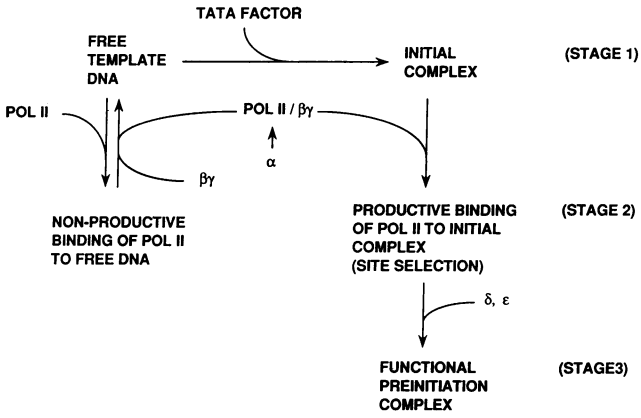

FIG. 5. Proposed mechanism for assembly of the functional preinitiation complex. POL II, RNA polymerase II.

complex by a mechanism similar to that utilized by bacterial σ factors. Several lines of evidence argue that, together, α and $\beta\gamma$ expedite site selection (*i*) by reducing the affinity of RNA polymerase II for nonpromoter sites in DNA, thereby preventing formation of nonproductive binary complexes of polymerase and free DNA, and (*ii*) by increasing the affinity of RNA polymerase II for the initial complex. First, we previously observed that $\beta\gamma$ suppresses the nonselective binding of RNA polymerase II to free DNA, most likely through a direct interaction with polymerase (15). In this respect, therefore, $\beta\gamma$ shares functional properties with *E. coli* σ^{70}, which interacts with *E. coli* RNA polymerase and reduces its affinity for nonpromoter sites in DNA (2, 3). Second, the results of restriction site protection analysis as well as previous template challenge experiments (13, 14) argue that both α and $\beta\gamma$ are required for stable binding of RNA polymerase II to the initial complex. Third, results of the template challenge experiments presented here strongly suggest that the target of α and $\beta\gamma$ is RNA polymerase II and not the initial complex. Although both factors interact stably and stoichiometrically with the preinitiation complex in the presence of RNA polymerase II, neither factor interacts stably with this complex in the absence of polymerase. These observations are most consistent with the model that α and $\beta\gamma$ promote site selection not by interacting with the TATA factor to reconstitute a recognition site for polymerase but, instead, by acting directly on polymerase itself. Exactly how α and $\beta\gamma$ interact with RNA polymerase II during assembly of the preinitiation complex is presently unknown. Despite the functional similarities between α and $\beta\gamma$ and bacterial σ factors, it is clear that neither α nor $\beta\gamma$ binds to RNA polymerase II as avidly as, for example, *E. coli* σ^{70} binds to its cognate RNA polymerase. Further evidence in support of the idea that $\beta\gamma$ interacts directly with RNA polymerase II comes from results indicating that $\beta\gamma$ is a likely homologue of the human transcription factor RAP30/74 (27), which has been shown to bind RNA polymerase II in solution. $\beta\gamma$ and RAP30/74 are both composed of two subunits of approximately 30 and 70 kDa each. More significant, antibodies directed against the 30-kDa subunit of RAP30/74 cross-react with the 30-kDa (γ) subunit of $\beta\gamma$ (K.P.G., unpublished data). Interestingly, Sopta *et al.* (27) recently reported that the 30-kDa subunit of RAP30/74 has sequence homology with bacterial σ factors.

Finally, it should be pointed out that the model for site selection proposed here is not inconsistent with the possibility that α, $\beta\gamma$, or both factors interact with the initial complex upon entry of RNA polymerase II into the preinitiation complex. Although it is clear, for example, that α does not interact stably with the initial complex in the absence of RNA polymerase II, under buffer and ionic strength conditions optimal for transcription *in vitro*, α, like TFIIB, may well interact with the initial complex under gel mobility-shift conditions, which have been reported to stabilize weak intermolecular interactions (28). Such weak interactions could play a role in the binding of RNA polymerase II to the initial complex. In studies of bacterial preinitiation complexes, both *E. coli* σ^{70} and *Bacillus subtilis* σ^{43}, which do not themselves bind DNA, have been observed to interact with promoter DNA when associated with RNA polymerase (1).

After binding of RNA polymerase II to the initial complex, transcription factors δ and ε promote assembly of the functional preinitiation complex (stage 3). The results of restriction site protection experiments argue that δ and ε enter the preinitiation complex at this stage and direct formation of stable protein–DNA contacts that anchor the transcription apparatus to core promoter sequences near the cap site. Buratowski *et al.* (8) recently reported that, in a HeLa cell transcription system, a chromatographic fraction containing transcription factor TFIIE promotes formation of stable protein–DNA contacts near the cap site of the AdML promoter during the final step in assembly of an RNA polymerase II preinitiation complex. Interestingly, TFIIE is likely the human homologue of ε; both factors have been shown to be composed of polypeptides of approximately 34 and 58 kDa (12, 29).

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