Analysis of the Molecular Mechanisms for the Species-specific Transcription of Drosophila and Human tRNA Gene Transcription Components

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The transcription of eucaryotic tRNA genes requires two factors IIIB and IIIC, in addition to RNA polymerase III, to reconstitute this process in vitro. We have examined the functional exchangeability of these components from Drosophila and human systems. The reconstitution of heterologous IIIB and IIIC components demonstrated that neither factor will functionally substitute for the homologous components to activate tRNA gene transcription. The addition of the heterologous Drosophila factors to HeLa transcription assays causes an inhibition of RNA synthesis that is dependent upon the order of addition of these proteins to the DNA template. Thus, it appears that tRNA gene transcription in these systems is species-specific. We have further analyzed the reason for the apparent incompatibilities of these components by the use of stable complex formation assays. We find that human HeLa IIIB and Drosophila IIIC are unable to form stably associated complexes with a tRNA gene template, whereas the Drosophila IIIB and HeLa IIIC do form stable but nonproductive complexes. These results demonstrate that specific IIIC-IIIB interactions are critical in the formation of productive transcription complexes and are responsible for the observed species specificity of Drosophila and human tRNA gene transcription.

RNA polymerase III is responsible for the transcription of a variety of small nuclear RNAs, including 5 S RNA and tRNA. For the transcription of tRNA genes, two intragenic control regions (ICRs) \(^1\) are required which correspond to the D and T loops in the resultant tRNA (Sharp et al., 1985; Geiduschek and Tocchini-Valentini, 1988). Analysis of the transcription components from yeast, mammalian, and insect systems has shown that in all systems, two protein fractions, designated transcription factors IIIB and IIIC, are required to reconstitute tRNA gene transcription. Although these factor fractions obtained from different systems fractionate similarly and appear to have similar functional properties, direct comparisons of their structural and functional features have not been rigorously examined. The protein factor, IIIA, is additionally required for transcription of 5 S RNA genes and has been extensively characterized from Xenopus laevis. Analysis of this protein from different amphibian species has revealed that the sequences and functional properties of these proteins are not well conserved (Gaskins et al., 1989).

Factor IIIC has been shown to bind directly and specifically to the two ICRs. Analysis of IIIC interaction with a variety of mutant tRNA genes has revealed that its interaction with the DNA is dependent upon nucleotides within the ICRs which are highly conserved in all tRNAs (Geiduschek and Tocchini-Valentini, 1988). Thus, the ICR promoter recognition domains of IIIC from different systems appear to be quite similar. In addition to the requirement of the ICRs for transcription of these genes, promoter strength can be modulated by 5'-flanking DNA sequences (Sharp et al., 1985; Geiduschek and Tocchini-Valentini, 1988), and in the insect systems, these sequences are essential for template activity in vitro (Sprague et al., 1980; Dingermann et al., 1982). Analysis of a Drosophila tRNA\(^{5'}\) gene revealed that the first 32 base pair flanking the gene were required for its transcription in Drosophila cell extracts (Schaack et al., 1984). In contrast, deletion of these 5'-flanking sequences has no effect on template activity in human HeLa cell extracts. Likewise, three Drosophila tRNA\(^{5'}\) genes exhibit different transcription efficiencies in Drosophila extracts which are dependent upon their 5'-flanking sequences (Loqquist and Sharp, 1986). These results suggest that differences may exist between the Drosophila and human RNA polymerase III transcription machinery which may dictate 5'-flanking sequence dependence.

Template competition assays have shown that IIIB and IIIC form stable transcription complexes with tRNA gene templates and remain bound through many rounds of transcription initiation (Schaack et al., 1983). IIIC binds to the template in the absence of IIIB, and the subsequent interaction of IIIB further stabilizes the IIIC-DNA complex (Lassar et al., 1983; Baker and Hall, 1984; Fuhrman et al., 1982; Johnson-Burke and Soll, 1985). In the yeast system, IIIB has been shown to act as an initiator factor to position RNA polymerase into the complex (Kassavetis et al., 1990). Although IIIB does not bind specifically to the template in the absence of IIIC, interaction of IIIB with the IIIC-DNA complex involves its interaction with the 5'-flanking region of both tRNA and 5 S RNA genes (Kassavetis et al., 1989). IIIC allows IIIB to be assembled into the complex and, once IIIB is bound, IIIC can be dissociated in the presence of heparin or high salt while IIIB remains stably bound to the DNA (Kassavetis et al., 1990). Another potential interaction in the formation of stable transcription complexes is the direct interaction of IIIB with IIIC. This was suggested by the initial analysis of the compatibility of the IIIB and IIIC components...
from heterologous systems (Burke and Söl, 1985). Reconstitution of Drosophila IIIB and human HeLa IIIC with a tRNA gene template did not produce transcription, suggesting that these components were unable to form productive transcription complexes. In the present study, we have further analyzed the functional exchangeability between the Drosophila and HeLa IIIB and IIIC components. Neither reconstitution of Drosophila IIIB and HeLa IIIC or reconstitution of HeLa IIIB and Drosophila IIIC is able to activate tRNA gene transcription. Using stable complex formation assays, we show that the inability of Drosophila IIIC and HeLa IIIB components to reconstitute tRNA gene transcription is a result of their inability to form stable transcription complexes. On the other hand, the inability of Drosophila IIIB and HeLa IIIC to activate transcription is a result of the formation of stable but nonproductive complexes. Thus, our results demonstrate that correct positioning of the protein-protein interactions between IIIB and IIIC play a major role in the formation of active transcription complexes and is responsible for the species specificity observed between the Drosophila and human RNA polymerase III systems.

**EXPERIMENTAL PROCEDURES**

**Plasmid DNAs**—The plasmid DNAs containing tRNA genes used in the experiments described are pArg, a Drosophila tRNA<sup>Arg</sup> gene (see Silverman et al., 1979); pArg 26x36a, a maxigene derivative of pArg which contains an additional 12 nucleotides inserted between the internal promoter regions (see Dingermann et al., 1983); pAsn8, a Drosophila tRNA<sup>Asn</sup> gene; and pAsn98, a maxigene derivative of pAsn8 which contains 20 additional nucleotides between the internal promoters (see Loqfist and Sharp, 1986).

**Transcription Assays**—Standard reactions were performed as described by Burke and Söl, 1985. The final reaction mixture contained 0.35 mg of DNA template, 20 mM Hapes, pH 7.9, 5 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 100 mM KCl, 10% glycerol, 0.5 mM each of ATP, CTP, UTP, and 0.1 mM [α<sup>32</sup>P]GTP (6 Ci/mmol) in 60 μl of final volume. Various factor fractions used in the reactions are indicated in the figure legends. Transcription reaction conditions, whereby a given factor fraction was limiting, were determined empirically for each protein preparation. Transcription reactions were incubated for 1 h at room temperature. All preincubation steps, where indicated, were also carried out at room temperature. The reactions were terminated by the addition of 0.5% sodium dodecyl sulfate and 10 μg/ml proteinase K. After incubation at 37°C for 15 min, RNAs were purified by phenol extraction and ethanol precipitation, and analyzed by electrophoresis on 8 M urea, 8% polyacrylamide gels. Gels were exposed to x-ray film for autoradiography.

**Extracts and Transcription Factor Preparation**—The Drosophila Schneider S-2 cell and human HeLa cell cytoplasmic (S100) extracts were prepared as described previously (Dingermann et al., 1981) and contained 5–10 mg of protein/ml. These extracts were subsequently fractionated on phosphocellulose (Whatman P-11) as described by Burke and Söl, 1985) with the following modification. For the preparation of the P-11 resin, base and acid washes were followed according to the manufacturer, and the column was next washed with three to four column volumes of buffer A (20 mM Hapes, pH 7.3, 0.1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol) containing 0.5 mg/ml bovine serum albumin and 0.1 M KCl. The column was subsequently washed with two column volumes of buffer A containing 1 M KCl and then re-equilibrated in buffer A containing 0.1 M KCl before loading the extract. This procedure allowed us to obtain reproducible results with different batches of phosphocellulose resin. Extracts were loaded onto P-11 columns at 5 ml/bed ml of bed volume. Protein concentrations were determined by the method of Bradford (1976). The column was washed with this buffer and proteins were step-down eluted with buffer containing 1 M KCl and then buffer A. The 0.35 M KCl fractions containing IIIB and RNA pol III activities was dialyzed against buffer A containing 5 mM MgCl<sub>2</sub> and 35 mM (NH₄)₂SO₄. The diazlyzed fraction was loaded onto a CM-Sepharose 6B column equilibrated in the same buffer. CM-Sepharose chromatography was carried out as described previously (Johnson-Burke and Soh, 1981). Protein fractions from the flow-through, which were dialyzed against buffer A containing 5 mM MgCl<sub>2</sub> and 0.1 M KCl and loaded onto a heparin-Sepharose column. The column was washed with buffer A containing 5 mM MgCl<sub>2</sub> and 0.275 M KCl, and IIIB was eluted with buffer containing 0.6 M KCl. This fraction contained no detectable RNA pol III or IIIC activity. RNA pol III activity was eluted from the CM-Sepharose column with 0.35 mM (NH₄)₂SO₄ in buffer A. This CM-Sepharose fraction was dialyzed against 60 mM KCl, loaded onto DEAE-Sepharose equilibrated in the same buffer, and RNA pol III activity was eluted with 1 M KCl in buffer A. For the HeLa RNA polymerase III used in Fig. 1, it was prepared by the procedure described by Kovelman and Roeder (1990), and was kindly supplied by Robert Kovelman (Rockefeller University, New York). For further fractionation of IIIC, the 0.7 M KCl step-eluted P-11 fraction containing IIIC and RNA pol III activity was dialyzed against buffer A containing 5 mM MgCl<sub>2</sub> and 35 mM (NH₄)₂SO₄ and loaded onto a CM-Sepharose 6B column equilibrated in the same buffer. The IIIC and RNA pol III activities were eluted with buffer A containing 0.35 mM (NH₄)₂SO₄. All resulting protein fractions used in the transcription assays were dialyzed against buffer A containing 5 mM MgCl<sub>2</sub> and 100 mM KCl. These fractions were supplemented with 0.5 mg/ml bovine serum albumin for stabilization of protein activity and stored at -70°C.

**RESULTS**

Drosophila and Human tRNA Gene Transcription Factors Do Not Reconstitute Transcription—In order to analyze the compatibility between Drosophila and human RNA polymerase III factors, we first examined whether the reconstitution of IIIB and IIIC from heterologous systems would produce specific transcripts. For the preparation of these components, cytoplasmic (S100) extracts were derived from Drosophila Schneider S2 cells and human HeLa cells (Dingermann et al., 1981). Factors IIIB and IIIC were subsequently separated from these extracts using phosphocellulose chromatography. The IIIC preparations derived from phosphocellulose chromatography were further purified using CM-Sepharose chromatography as previously described and also contained RNA polymerase III activity (Burke et al., 1983). Since our phosphocellulose-derived IIIB preparations occasionally contained contaminating amounts of IIIC (as judged by its ability to reconstitute low levels of transcription independently of the added IIIC fractions), we further fractionated IIIB on CM-Sepharose and heparin-Sepharose column chromatographies which effectively removed any residual IIIC activity as well as RNA polymerase III (see “Experimental Procedures”). As an additional criterion for the purity of the IIIB fractions, we measured their ability to be sequestered onto a tRNA gene template using a stable complex formation assay. These more purified IIIB fractions, which did not contain the ability to bind to a tRNA gene template in the absence of the IIIC fraction, were used for subsequent analysis.

As shown in Fig. 1, none of the Drosophila or HeLa factor fractions alone supports transcription of a Drosophila tRNA<sup>Arg</sup> gene template (lanes 1–4). The reconstitution of the homologous HeLa or Drosophila IIIB and IIIC components was able to efficiently transcribe relatively similar levels from the tRNA<sup>Arg</sup> gene template (lanes 5 and 6). The various RNAs generated in these assays are a result of differential processing of the precursor transcripts, which varied, and the Drosophila and HeLa factor preparations. The reconstitution of heterologous Drosophila IIIB (dIIIB) and HeLa IIIC (hIIIC) or HeLa IIIB (hIIIB) and Drosophila IIIC (dIIIC) factors was unable to activate tRNA gene transcription (lanes 7 and 9). Our previous studies showed that the reconstitution of hIIIB and dIIIC was able to reconstitute low levels of transcription activity relative to the reconstitution of the homologous factors (Burke and Söl, 1985). This discrepancy is most likely a result of the purity of the hIIIB fraction used. Our initial hIIIB preparations were less rigorously purified and although they alone did not support specific transcription, further analysis of these fractions revealed that they still contained...
detectable levels of specific DNA binding activity (data not shown). In the present study, the reconstitution of more highly purified IIIB with heterologous IIIC components did not allow the generation of any detectable specific tRNA gene transcripts. Since the IIIC fractions contained the RNA polymerase III activity, it was possible that in order to reconstitute transcription with the heterologous factors, the RNA polymerase may have to be derived from the same system as IIIB. To test this possibility, the dIIIB and hIIIC fractions were reconstituted with purified HeLa RNA polymerase III; HB, HeLa IIIB; HC, HeLa IIIC; and HP, HeLa RNA polymerase III. Transcription products were resolved by gel electrophoresis and detected by autoradiography. The bracket indicates the precursor and processed tRNA^Ala transcripts generated.

**FIG. 1. Drosophila and human tRNA gene transcription is species-specific.** Factors IIIB and IIIC were prepared from Drosophila and HeLa cell cytoplasmic extracts, and in vitro transcription assays were performed as described under "Experimental Procedures." Each assay contained 0.75 µg of pArg DNA as template. The fractions included or omitted in each reaction are indicated by + or − above each lane and are designated as DB, Drosophila IIIB; DC, Drosophila IIIC; DP, Drosophila RNA polymerase III; HB, HeLa IIIB; HC, HeLa IIIC; and HP, HeLa RNA polymerase III. Transcription products were resolved by gel electrophoresis and detected by autoradiography. The bracket indicates the precursor and processed tRNA^Ala transcripts generated.

**FIG. 2. Drosophila factors IIIB or IIIC inhibit tRNA gene transcription of reconstituted human factors IIIB and IIIC.** In vitro transcription assays were performed as described under "Experimental Procedures" using 0.75 µg of pAsn8 as template. The fractions in each reaction are indicated by + or − . The triangles indicate varying concentrations of added HeLa IIIB or IIIC. In panel A, 5 µg of hIIIB and, where indicated, 4 µg of dIIIC protein fractions were used. In addition, the following limiting of concentrations of hIIIB fractions were added: 2 µg (lanes 1 and 4), 4 µg (lanes 2 and 5), and 6 µg (lanes 3 and 6). In panel B, 3.5 µg of hIIIC and, where indicated, 2 µg of dIIIB protein fractions were used. In addition, the following limiting of concentrations of hIIIC fractions were added: 1 µg (lanes 7 and 10), 2 µg (lanes 8 and 11), and 4 µg (lanes 9 and 12). Brackets indicate that tRNA^Ala gene transcripts generated from each reaction.

**FIG. 3. The extent of inhibition of tRNA gene transcription by Drosophila IIIB or IIIC is dependent upon the order of addition of the transcription components.** In vitro transcription assays were performed using 0.75 µg of pAsn8 as template and the factor fractions indicated by + or − . The triangles indicate varying concentrations of added factors. The factor fraction was preincubated with the DNA template for 40 min prior to the addition of other factor fractions and NTPs. Brackets, precursor and processed tRNA transcripts generated.

**Drosophila IIIB or IIIC Specifically Inhibits Human tRNA Gene Transcription**—In order to further address the possible functional incompatibility of the Drosophila and HeLa RNA polymerase III components, we examined whether the lack of detectable RNA products in the heterologous reconstituted fractions was just due to nonspecific inhibitors or nucleases contained in these fractions. To address the possibility, we examined whether the Drosophila factors would inhibit transcription in the homologous HeLa systems. As shown in Fig. 2A, addition of dIIIC to assays containing hIIIB and hIIIC fractions showed a reduction in the level of transcription compared to assays containing just the HeLa factors (compare lanes 1–3 to 4–6). Likewise, the addition of dIIIB to assays containing the hIIIB and hIIIC factors also decreased transcription activity of the reconstituted homologous factors (Fig. 2B, compare lanes 7–9 and 10–12). We examined whether the addition of increasing amounts of the homologous HeLa factors to the reactions could relieve the inhibition by the Drosophila fractions. In the presence of dIIIC, the addition of increasing amounts of hIIIC was able to overcome the inhibition observed (panel A). Likewise, by adding increasing amounts of hIIIB to reactions containing dIIIB, transcription was also proportionally increased (panel B). Since it had been previously shown that high concentrations of hIIIB in transcription reactions could be inhibitory (Waldschmidt et al., 1988), these results show that the inhibition we observed is not simply due to an over-titration of IIIB in the reactions. These results suggest the Drosophila fractions do not contain nonspecific inhibitors or nucleases which are preventing or masking transcription reconstitution of the heterologous components.

To further assess that the decreased transcription levels were a result of specific inhibition by the Drosophila factors, we varied the time of addition of the Drosophila and HeLa factors with the tRNA gene templates. Since the ability of IIIC and IIIB to form stable transcription complexes with the template is a general feature of these components, we reasoned that if either HeLa or Drosophila factors are preincubated with the template to allow for the formation of stable complexes, the order of their addition should change the levels of transcription. Examining the addition of dIIIC to transcription assays containing hIIIB and hIIIC factors, when dIIIC, hIIIB and hIIIC were simultaneously added, decreased transcription was observed relative to the HeLa factors alone (Fig. 3A, lanes 1 and 2). The preincubication of hIIIB and hIIIC components with the template prior to the addition of dIIIC resulted in an increase in transcription (lane 3). On the other hand, the prior incubation of hIIIB and dIIIC with the template and subsequent addition of hIIIC resulted in a more dramatic decrease in transcription (lane 4). These results suggest that when the homologous components are allowed to be sequestered onto the template forming stable transcription complexes the dIIIC fraction has a decreased ability to inhibit transcription. When dIIIC is preincubated and allowed to bind to the DNA, prior to adding hIIIC, it increases the inhibition of transcription observed. Thus, the level of tran-
cription observed is dependent upon which IIIC component is allowed to bind first to the template.

We next examined the order of addition of either dIIIB or hIIIB to the template and hIIIC (Fig. 3B). When the homologous components are preincubated with the template prior to the addition of dIIIB, the Drosophila fraction has little affect on the transcription activity compared to its simultaneous addition with the HeLa factors and template (lanes 6 and 7). However, when dIIIB is preincubated with hIIIC and the template and hIIIB is added later, its inhibiting activity is enhanced relative to the simultaneous addition of all three factors (lane 8). These results suggest that the inhibiting activity is specifically a result of the IIIB component in the Drosophila fraction. The effect of the order of addition of these fractions on the level of inhibition further substantiates that the incompatibilities observed are not a result of incompatibilities of the RNA polymerase. The increased inhibition observed when dIIIB is allowed to preincubate with hIIIC and template additionally suggests that it may form a stable but nonproductive association with the hIIIC-template complex.

Thus, these results showed that (1) increasing the amounts of the corresponding homologous HeLa components to the transcription assays could overcome the inhibitory effect of the Drosophila factor fractions, and (2) an enhancement or reduction in the inhibitory effects of the Drosophila fractions was observed when the order of addition of the Drosophila and HeLa components to the template was varied. Taken together, these data support the observation that the HeLa and Drosophila IIIB and IIIC components do not reconstitute tRNA gene transcription due to an incompatibility between these factors.

**Drosophila IIIC and Human IIIB Do Not Form Stable Preinitiation Complexes with a tRNA Gene Template**—To further examine the reason for the apparent species-specific transcription observed for the Drosophila and HeLa RNA polymerase III components, we next analyzed the ability of the heterologous factors to form stable complexes. Previous studies of both the human (Lassar et al., 1983) and Drosophila (Burke and Soll, 1985) transcription systems have demonstrated that IIIC directly interacts with the DNA template and the subsequent interaction of IIIB with the IIIC-DNA complex results in the formation of a stable transcription complex. This complex remains associated for many rounds of transcription initiation. The interaction of IIIB with the IIIC-DNA complex is thought to occur through both IIIB-IIIC and IIIB-DNA contacts although, so far, in the yeast system more direct evidence exists for IIIB-DNA contacts (Kassavetis et al., 1988, 1989). Since IIIC proteins analyzed from such diverse systems as yeast, Xenopus, insect, and mammalian cells all appear to recognize common consensus sequences within the tRNA gene ICRs (Sharp et al., 1985; Geiduschek and Tocchini-Valentini, 1988), the Drosophila and HeLa IIIC share a common DNA binding specificity and, presumably, similar DNA-binding domains. Therefore, the inability of the heterologous components to form productive transcription complexes indicates that there is structural and functional dissimilarity in the protein-protein interaction domains of these factors. Two possibilities could, therefore, exist. 1) The heterologous IIIB and IIIC factors do not form stable complexes necessary for transcription initiation, or, 2) the heterologous IIIB and IIIC factors do form stable complexes, albeit aberrant ones, which do not allow RNA polymerase to initiate transcription. To analyze each possibility, we examined the abilities of either hIIIB and dIIIC or dIIIB and hIIIC to form stable complexes using template commitment assays.

We first analyzed the ability of hIIIB and dIIIC to form stable complexes. In the first experiment, we measured the ability of IIIB to bind stably to a IIC-DNA complex by preincubating hIIIB, and either hIIIC or dIIIC, and the tRNA<sub>Asn</sub> gene (DNA1) for varying periods of time (Fig. 4A). To analyze whether hIIIB was bound to the IIC-template complex, a second template, a maxigene derivative of the tRNA<sub>Asn</sub> gene (DNA2), was added which had itself been preincubated with hIIIC. If IIIB forms a stable complex with IIIC and the first template, at increasing incubation times hIIIB will be sequestered and less available for transcribing the IIIC-DNA2 complex added later. As shown in Fig. 4A, hIIIB, hIIIC, and the tRNA<sub>Asn</sub> gene template (DNA1) were preincubated for varying periods of time, and then the

![Figure 4](https://example.com/figure4.png)

**FIG. 4.** Human IIIB and Drosophila IIIC do not form stable preinitiation complexes with a tRNA gene template. A, human IIIB is not sequestered into a stable complex with Drosophila IIIC. The experimental protocol is outlined at the top. 0.3 μg of pAsn<sub>8</sub> template (DNA1) and a limiting amount, 3 μg hIIIB, was included in the preincubation mixture (Mix1) together with either 4 μg of hIIIC or 4 μg of dIIIC as indicated. The preincubations were carried out for 0, 10, 20, 40, or 60 min as indicated above each lane. After the preincubation step, Mix 2 and NTPs were added. Mix 2 contained 0.3 μg of pAsn<sub>8</sub> (DNA2) and 4 μg of hIIIC which had been preincubated 15 min prior to its addition to the reaction. After Mix1, Mix2, and NTPs were combined, the reaction was allowed to proceed for 60 min. tRNA gene transcripts generated from DNA1 and DNA2 are designated by the reactions: dIIIB, hIIIC, and the following limiting amounts of IIIB fractions: no addition (lanes 1-4); 0.4 μg lane 5 and 7; 0.8 μg lane 6 and 8; 1.5 μg lane 9, 11, and 3 μg lane 12. After the preincubation step, the following limiting amounts of IIIB fractions (Mix2) were added to the reaction: dIIIB, 0.4 μg (lanes 1 and 5); 0.8 μg (lanes 2 and 6); 1.5 μg (lanes 3 and 7); 3 μg (lanes 4 and 8); and 5 μg of hIIIB was added to lanes 9-12. After the addition of Mix2, NTPs were added and the reaction was allowed to proceed for 60 min. The tRNA<sub>Asn</sub> gene transcripts generated from each reaction are designated by the arrow.
tRNA\textsuperscript{A\texttextsuperscript{m}} maxigene (DNA2) was added after it was preincubated with hIIIC. As expected, the homologous hIIIB and hIIIC components are able to be sequestered onto the first template with increasing preincubation time, reducing transcription of the second IIC-DNA2 complex added later (lanes 1–5). In contrast, when hIIIB is preincubated with dIIIC and the template, even after prolonged incubations, equal amounts of transcription are produced from the second template (lanes 6–10). Thus, these results suggest that under conditions where the homologous HeLa factors form stable complexes, hIIIB appears unable to form a stable complex with the dIIIC and DNA template.

The previous experiment examined the ability of hIIIB to be stably sequestered onto the dIIIC-template complex by analyzing transcription of a reference template added later. Additionally, if the dIIIC is not complexed with hIIIB, then its protein-binding site should be available for interacting with subsequently added dIIIB. We, therefore, analyzed the availability of the dIIIC-template to form productive transcription complexes with dIIIB either before or after preincubation with hIIIB. As shown in Fig. 4B, preincubation of dIIIC with the tRNA\textsuperscript{A\texttextsuperscript{m}} gene template and subsequent addition of increasing amounts of dIIIB produces a corresponding increasing in the amounts of transcript (lanes 1–4). When hIIIB is preincubated with the template and dIIIC before the addition of increasing amounts of dIIIB, the same levels of transcription are observed (compare lanes 1–4 to lanes 5–8). Changing the order of addition of dIIIB and hIIIB, similar levels of transcription are observed (compare lanes 5–8 and lanes 9–12). Thus, regardless of whether hIIIB is present or absent in the preincubation step, the dIIIC is able to form relatively the same amounts of productive transcription complexes with dIIIB. We interpret this to mean that the IIIB-binding site on the IIIC protein is not stably bound by hIIIB and is therefore available to interact with dIIIB. Taken together, these results indicate that hIIIB is unable to form a stable preinitiation complex with dIIIC and a tRNA gene template.

Drosophila IIIB and HeLa IIC Are Able to Form Stable Nonproductive Preinitiation Complexes with the tRNA Gene Template—We next analyzed the ability of dIIIB and hIIIC to form stable complexes. As shown in Fig. 5A, using the two template commitment assay, when dIIIB was preincubated with hIIIC and the tRNA\textsuperscript{A\texttextsuperscript{m}} gene for increasing periods of time, decreasing levels of transcription resulted from the maxigene reference template (DNA2) (lanes 6–10). The time course observed for the reduction in the maxigene template transcription paralleled that observed when both of the homologous HeLa components were used (lanes 1–5). These results indicate that the dIIIC component is capable of forming a stable complex with hIIIC and the template. To further ascertain this possibility, we next measured the availability of hIIIC to form a complex with the hIIIB in the presence dIIIB. We reasoned that if the dIIIB factor is interacting specifically with hIIIC at its IIIB interaction site, when hIIIB is subsequently added to the dIIIB-hIIIC-template complex, it will be unable to interact with hIIIC and have little resultant effect on the amount of transcription produced. As shown in Fig. 5B, when dIIIB, hIIIC, and the template are preincubated for 40 min to allow for complex formation, the subsequent addition of increasing amounts of hIIIB yields only low levels of transcription products (lanes 1–3). When hIIIB is preincubated first and dIIIB is added later (lanes 4–6) or buffer alone is added later (lanes 10–12), hIIIB is able to stimulate relatively high levels of transcription. Preincubation of hIIIC alone with the template prior to addition of hIIIB also showed increased levels of transcription (lanes 7–9) compared to their preincubation with dIIIB (lanes 1–3). Thus, these results demonstrate that dIIIB and hIIIC components do form stable complexes. Since this interaction prevents hIIIB from binding to the hIIIC-template complex, this further suggests that the binding of dIIIB to hIIIC occurs at, or in close proximity to, its specific IIIB-binding domain.

**DISCUSSION**

Our results demonstrate that the *Drosophila* and human RNA polymerase III transcription components B and C are species-specific. We find that the two DNA-binding transcription factors, *Drosophila* IIC and human IIC, cannot be exchanged in transcription assays even though they contain
the same DNA promoter recognition properties. These results indicate that there are differences in the putative IIIB protein interaction domains of these two IIIC proteins that are responsible for their inability to activate tRNA gene transcription in heterologous assays. The class III genes that only require the transcription factors IIIB and IIIC include tRNA genes and viral-associated genes such as VA1 and VA2 RNA genes from adenovirus. The two ICRs are highly conserved in these genes, and correspond to the D and T regions in the resultant RNAs. Since these regions contain nucleotides that are essential for tRNA function, extensive sequence divergence of these elements within these genes has not occurred. Therefore, specific IIIC-DNA interactions involved in transcription of these genes has also presumably been maintained through evolution. Despite the apparent conservation of promoter elements and IIIC-DNA interactions, the IIIB-IIIC protein interactions appear to have changed significantly.

Analyzing the reconstitution of heterologous hIIIB and dIIIC components, we find that they are unable to form a stable complex. Although we cannot rule out the possibility that these proteins do associate, our results demonstrate that under conditions that produce a stable association of either of the homologous components with the DNA template, these factors do not. It is possible that protein contacts do occur but that the "off-rate" of hIIIB with the dIIIC-DNA complex is greater than what is observed in either of the homologous systems. The reconstitution of the dIIIB and hIIIC components with the tRNA gene template resulted in the formation of stable, but nonproductive, complexes. This finding suggests that the stable binding of IIIB to the IIIC-DNA complex is insufficient for transcription initiation to occur. The insufficiency of IIIB to the stable complex containing IIIB, hIIIC, and the DNA template, suggests that dIIIB binds to the IIIC factor at a site which is in close proximity to the normal IIIB-binding site, rendering it sterically unable to bind additional hIIIB. This indicates that proper positioning of the IIIB protein on the complex is critical and plays a major role in specifying tRNA gene activity in humans and Drosophila.

The formation of stable preinitiation complexes is a general feature of class III genes from all systems studied (Palmer and Folk, 1990). By using template competition assays and limiting factor concentrations, the order in which these components assemble onto tRNA gene templates to form stable complexes has been examined. For these genes, the association of IIIC is the first step in complex formation, and the subsequent interaction of IIIB stabilizes this interaction (Lascar et al., 1983; Burke and Soll, 1985). Human IIIC has been chromatographically separated into two fractions, designed factors IIIC1 and IIIC2 which will reconstitute tRNA gene transcription in the presence of IIIB and RNA polymerase III (Dean and Berk, 1987; Yoshinaga et al., 1987). Detailed analysis of the ordered interactions of these components in the formation of stable complexes on tRNA and VA RNA gene templates has revealed that IIIC2 binds directly and stably to these templates. After IIIC2 is bound to the DNA, either IIIB or IIIC1 can then interact to form a preinitiation complex (Dean and Berk, 1988). The identification of the IIID factor from the Bombyx mori system (Ottolento et al., 1987), which appears to be functionally analogous to either IIIC1 or IIIC2 from the human system, raises the possibility that Drosophila IIIC may also contain two separable components. Analysis of complex formation with the B. mori tRNA^Ala gene revealed that in this case, all three B. mori components, IIIB, IIIC, and IIID, were necessary for template commitment. It is possible that for the productive interaction of dIIIB with the hIIIC-DNA complex, both IIIB-IIIC1 and IIIB-IIIC2 interactions need to occur. Although the interaction of IIIB with IIIC2 may be sufficient for the formation of a stable complex, the additional interaction of IIIB with IIIC1 may be required for initiation of transcription. Thus, the correct positioning of IIIB on the complex could be dependent upon more than one protein-protein contact with IIIC. The inability of dIIIB and hIIIC tRNA gene complexes to initiate transcription could be a result of improper positioning of IIIB onto the IIIC-DNA complex or due to the lack of one of the required protein-protein contacts with the IIIC-DNA complex. Clearly, defining the complex set of protein-protein interactions that specify the interaction of IIIB with the transcription complex awaits direct physical characterization of these protein and their associations.

In the yeast system, extensive analysis of IIIB interactions with the IIIC-tRNA gene template complex by DNase-1 protection assays has revealed that in the presence of IIIB, the protected region of the DNA is extended compared to that observed for IIIC-DNA interactions. In the presence of IIIB, protection occurs through the 5' flanking sequence of the gene directly upstream of the transcription start site (Kassaves et al., 1989). This suggested that, although IIIB alone does not interact directly and specifically with the template (Klemp and WelI, 1986), it may directly contact the DNA through an IIIC-dependent association. This possibility was further substantiated by the finding that hIIIC and high salt conditions were able to selectively remove IIIC from the IIIB-IIIC-DNA complex (Kassaves et al., 1990). Under these conditions, IIIB remained bound to the DNA and was capable of initiating transcription in the presence of RNA pol III with the same efficiency as when IIIC was present. These results reveal that once IIIB is assembled into the complex by IIIC, IIIB functions as the sole transcription initiator factor. This corroborates our current studies which indicate that the proper positioning of IIIB into the complex is critical for initiation to occur. Thus, it appears that both the direct interaction of IIIB-IIIC as well as IIIB-DNA interactions specify the assembly of IIIB onto active tRNA gene transcription complexes.

Analysis of the mechanisms that govern eucaryotic transcription is increasingly implicating protein-protein interactions as playing critical roles in specifying these processes as well as their regulation. The species-specific nature of RNA polymerase I transcription has been well-documented (Soller-Webb and Towner, 1986). Early studies revealed that unlike RNA polymerase II and III, the RNA polymerase I transcription machinery would only recognize tRNA promoters from closely related species (Grummt et al., 1982). Since the rRNA promoters are not highly conserved between different eucaryotic systems, the species-specific nature of these systems was thought to be a result of protein-DNA interactions which conferred differential promoter selectivities. The unexpected observation that the DNA-binding RNA polymerase I factor, UBF, from human and Xenopus systems were both able to bind to the human RNA polymerase I promoter, allowed re-evaluation of the mechanism for species-specific transcription (Bell et al., 1989). These results demonstrated that the subsequent interactions of the RNA polymerase I protein components with UBF-DNA complexes play a major role in specifying RNA polymerase I promoter activity. Thus, protein-protein interactions appear to be quite stringent in both the RNA polymerase I and III systems and are responsible for the species-specific nature of these transcription processes. In contrast, RNA polymerase II transcription factors are found to be functional for a large variety of promoters across species (Mitchell and Tjian, 1989). Analysis
of the general transcription machinery from Drosophila and HeLa systems (including IIB, IID, IIE, and RNA polymerase II) has revealed that these components are compatible and are able to be functionally exchanged in transcription reconstitution assays (Wampler et al., 1990). Thus, in the RNA polymerase II system, both in general protein factors, as well as the regulatory factors, appear to be quite flexible in protein-protein interactions required for transcription activation.

Further comparisons of the RNA polymerase III transcription machinery from mammalian and insect systems will not only help to delineate the critical protein-protein interactions which specify transcription, but will allow us to assess potential differences in the interactions which could lead to the differential requirements for 5'-flanking DNA sequences observed. It is possible that these differences could ultimately affect the manner in which gene regulation occurs in these systems.

Acknowledgements—We would like to thank Mitchell Garber for many helpful discussions and Chi Dang, Stephen Sharp, and Michael Stallcup for critical reading of the manuscript. Marcie Castaneda is gratefully acknowledged for preparation of the manuscript, and we thank Sam Griffin for his assistance with graphics. Robert Kovelman is acknowledged for his gift of HeLa RNA polymerase III and Arger Drew for HeLa cells.

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