CIF, an essential cofactor for TFIID-dependent initiator function

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The core promoters for mammalian protein-coding genes often contain a TATA box, an initiator (Inr) element, or both of these control elements. The TFIID complex is essential both for TATA activity and for the activity of a common class of Inr elements characterized by an approximate consensus sequence PyPyA₊₁NT/APyPy. Although the complete set of proteins required for basal TATA-mediated transcription has been established, the requirements for TFIID-dependent Inr activity remain undefined. In this study we set out to reconstitute Inr activity with purified and recombinant general transcription factors. For this analysis, Inr activity was measured as the ability of an Inr to enhance the strength of a core promoter containing an upstream TATA box. Inr activity was not detected in reactions containing TFIIB, RAP30, RAP74, RNA polymerase II, and either TBP or TFIID, even though these factors were sufficient for TATA-mediated transcription from supercoiled templates. By use of a complementation assay, a factor that imparts Inr activity was identified. This factor, named CIF, stimulated Inr activity in reactions containing the TFIID complex, but activity was not detected with TBP. Further characterization of CIF suggested that it contains multiple components. Functional and immunological experiments demonstrated that one of the CIF components is the mammalian homolog of *Drosophila* TAF_{II}150, which is not tightly associated with mammalian TFIID. These results reveal significant differences in the factor requirements for basal TATA and Inr activity. Further elucidation of these differences is likely to explain the need for the core promoter heterogeneity found within protein-coding genes.

[Key Words: TFIID; CIF; TATA box; transcription; Inr; TAF]

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Since the development of cell-free extracts that support accurate, promoter-dependent transcription by mammalian RNA polymerase II (Weil et al. 1979; Manley et al. 1980), much has been learned about the factor requirements for the transcription initiation event (Conaway and Conaway 1993; Drapkin et al. 1993). For specific initiation from TATA-containing promoters, the multisubunit polymerase must be supplemented with the TATA-binding protein (TBP), TFIIB, and TFIIF (composed of RAP30 and RAP74); in some cases, TFIIA, TFIIE, and TFIIH are also essential. In an in vitro reaction performed with purified components, these factors assemble onto the template in an ordered fashion, forming a preinitiation complex that is competent for the initiation of RNA synthesis following ATP hydrolysis and template melting.

Despite our extensive knowledge of the proteins re-

quired for TATA-mediated transcription, the requirements for initiation from promoters that lack a TATA box remain poorly defined. One type of TATA-lacking core promoter contains an initiator (Inr) element, a TATA analog that overlaps the transcription start site (Kollmar and Farnham 1993; Smale 1994). An Inr can be defined as a core promoter element, as it is capable of determining the location of the start site in a promoter that lacks a TATA box. A second characteristic activity of an Inr is the ability to enhance the strength of a promoter that contains a TATA box, if the Inr is located at the correct spacing (~25 bp) relative to TATA. Extensive analyses of randomly generated mutants have revealed that sequences matching a loose consensus of PyPyA₊₁NT/APyPy are capable of imparting both of these activities (Javahery et al. 1994; K. Lo and S.T. Smale, in prep.).

Studies from several laboratories have shown that the TFIID complex, which contains TBP and several TBP-associated factors (TAFs; Hernandez 1993), is required for efficient activity of Inr elements that match the

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above consensus (Smale et al. 1990; Conaway et al. 1991; Pugh and Tjian 1991; Zhou et al. 1992; Kaufmann and Smale 1994; Martinez et al. 1994). Furthermore, highly purified *Drosophila* and human TFIID complexes bind to consensus Inr elements, with the TFIID/Inr interaction apparently dependent on the precise nucleotides needed for Inr function (Kaufmann and Smale 1994; Purnell et al. 1994). In *Drosophila*, the TAF_{II}150 component of TFIID has been implicated as a potential candidate for mediating Inr recognition, and a trimeric TBP–TAF_{II}250–TAF_{II}150 subcomplex has been shown to support Inr activity when added to in vitro reactions in place of the complete TFIID complex (Sypes and Gilmour 1994; Verrijzer et al. 1994, 1995). Purified human TFIID complexes that recognize Inr elements and that support Inr activity, however, lack a detectable homolog of the *Drosophila* TAF_{II}150 protein (Zhou et al. 1992; Kaufmann and Smale 1994; Martinez et al. 1994). Thus, the Inr binding and stimulatory activities observed with purified human TFIID must be mediated either by a TAF_{II}150 homolog that is present in substoichiometric levels or by a different component of TFIID.

Although the TFIID complex appears to be involved in the recognition and function of consensus Inr elements, other Inr-binding proteins have been identified that stimulate transcription initiation in the presence of the isolated TBP subunit of TFIID or in the complete absence of either TBP or TFIID. TFII-I, for example, binds to Inr sequences in some promoters and directly recruits TBP to an upstream TATA box (Roy et al. 1991, 1993; Du et al. 1993). Another protein, YY1, binds with high affinity to Inr elements containing a CCAT core sequence and directs transcription initiation in the absence of TBP or TFIID (Seto et al. 1991; Usheva and Shenk 1994). Finally, RNA polymerase II has an intrinsic affinity for Inr sequences, leading to preinitiation complex formation when supplemented with TBP, TFIIB, and TFIIF (Carcamo et al. 1991; Aso et al. 1994). These three TFIID-independent mechanisms of transcription initiation appear to be distinct from the TFIID-dependent mechanism observed by us and others, which is the topic of the study described here.

The recognition of both TATA and Inr elements by the TFIID complex suggests that the two elements direct transcription initiation through similar mechanisms. This model is supported by several observations. For example, experiments performed with partially purified fractions have suggested that Inr-mediated transcription requires many of the general transcription factors required for TATA-mediated transcription (Pugh and Tjian 1991; Martinez et al. 1994). Furthermore, analyses of biochemical parameters of the initiation event, including Sarkosyl sensitivity and open complex formation, indicate that late steps during Inr-mediated transcription are similar to those that occur during TATA-mediated transcription (Zenzie-Gregory et al. 1992; Jiang et al. 1993; Martinez et al. 1994).

Despite the apparent similarities between TATA- and Inr-mediated transcription, recent studies have established that transcriptional activation domains and natu-

ral promoters can possess strong preferences for core promoters containing Inr elements. The glutamine-rich activation domain of Sp1, for example, stimulates transcription from Inr-containing core promoters much more strongly than from core promoters that lack an Inr element (Emami et al. 1995). In addition, the function of the lymphoid-specific murine terminal transferase promoter is absolutely dependent on its Inr element; when the Inr was mutated and a TATA box inserted at the –30 region, promoter activity was undetectable (Garraway et al. 1996). These results suggest that the mechanism of Inr-mediated transcription, as well as the factor requirements, may actually be quite different than for TATA-mediated transcription.

To analyze in detail the mechanism of Inr-mediated transcription and its relationship to the mechanism of TATA-mediated transcription, the minimal factor requirements for Inr activity must be defined. As a first step toward this goal, we analyzed Inr activity in the presence of recombinant and highly purified general transcription factors. We used basal promoters containing either a wild-type or mutant Inr element supplemented with a TATA box to ensure a detectable signal (see below). We tested the minimal factors known to be required for TATA-mediated transcription from a supercoiled template [RNA polymerase II, TBP, TFIIB, TFIIF(RAP30/74)] (Parvin and Sharp 1993; Tyree et al. 1993; Goodrich and Tjian 1994) and then substituted highly purified human TFIID complex for the recombinant TBP. Our results confirm the importance of the TFIID complex but suggest that TFIID and the minimal general factors are not sufficient for Inr activity. An additional activity that is not needed for TATA-dependent transcription has been partially purified based on its ability to stimulate Inr activity in the presence of the minimal general transcription factors. This activity, called CIF, (co factor of Inr function) appears to depend on multiple protein components, one of which appears to be the human homolog of *Drosophila* TAF_{II}150.

Results

Experimental strategy

Our laboratory and others have observed efficient, TFIID-dependent Inr activity with nuclear extracts or crude column fractions containing the known general transcription factors (Smale et al. 1990; Pugh and Tjian 1991; Zhou et al. 1992; Kaufmann and Smale 1994; Martinez et al. 1994), but not with highly purified proteins. To study the precise requirements for TFIID-dependent Inr activity, we set out to establish a minimal in vitro reconstitution assay using purified and recombinant general factors.

For this analysis, we measured specific transcription from synthetic promoters containing the terminal deoxynucleotide transferase (TdT) Inr [which matches the Inr consensus (Javahery et al. 1994)] in the context of a strong TATA box. Inr activity was defined as the difference between the strength of a promoter containing a

TATA box upstream of the wild-type TdT Inr versus the strength of similar promoters containing a mutant Inr. We did not employ promoters containing only an Inr in a TATA-less context because basal transcription from these promoters is weak and the transcription signals are extremely difficult to detect. [Basal transcription from the natural TdT promoter is quite strong, but the strong activity depends on the Inr as well as a second basal element downstream of the Inr that remains to be characterized (see Smale and Baltimore 1989).] Furthermore, we did not perform experiments to reconstitute Inr activity from a TATA-less promoter that depends on an upstream activator because transcription would depend on the use of factors (e.g., USA) that would add an additional level of complexity. Therefore, our strategy is to elucidate the factor requirements for Inr activity in the presence of a TATA box. Knowledge gained from these studies will facilitate future studies of the factor requirements for activated transcription from promoters that lack a TATA box.

For the assays described below, a 180-bp "G-less cassette" was inserted downstream of the wild-type and mutant promoters, and the promoter activities were measured by direct incorporation of [α - 32 P]UTP into RNA transcripts by use of standard in vitro transcription conditions (see Materials and methods). Correctly initiated transcripts were visualized by autoradiography after electrophoresis on denaturing polyacrylamide gels (Parvin and Sharp 1991; see Materials and methods). The three mutant promoters used for this analysis contain either a C at the +3 position (+3C), an A at the -1 position (-1A), or both of these substitutions (+3C-1A; numbering is relative to the +1 adenosine within the TdT Inr sequence, CCCTCATTCT). All three mutations reduce promoter strength 5- to 10-fold in transcription assays performed with crude nuclear extracts (see Fig. 1B,C).

For the reconstitution experiments, the basal factors TBP, TFIIB, RAP30, and RAP74 were purified following expression of the recombinant proteins in *Escherichia coli* (see Materials and methods). Epitope-tagged TFIID was purified by immunoaffinity chromatography (Fig. 1A; see Materials and methods; Kaufmann and Smale 1994). RNA polymerase II was partially purified by conventional chromatography or, for some experiments, was purified to near homogeneity by immunoaffinity chromatography (see Materials and methods).

The purified and recombinant transcription factors that reconstitute TATA-mediated transcription are not sufficient for Inr activity

The experiments presented in Figure 1B (lanes 4–9) demonstrate that TATA-mediated transcription can be detected in reactions containing a supercoiled template, partially purified RNA polymerase II, recombinant TFIIB, RAP30, RAP74, and either 10 or 40 ng of recombinant TBP. Similar results were obtained when immunoaffinity-purified RNA polymerase IIA was used instead of the partially purified polymerase (data not

shown). With both polymerase preparations, the transcription signals were greatly reduced or abolished when any of the individual factors were omitted (data not shown). The ability of these five proteins to support TATA-mediated transcription is consistent with results published previously by other laboratories (Parvin et al. 1993; Tyree et al. 1993; Goodrich and Tjian 1994).

Interestingly, the Inr did not appear to be active in the reconstituted transcription reactions described above. The inactivity was evident from the fact that the promoter containing the wild-type Inr was not significantly stronger than the promoters containing either the +3C, -1A, or +3C-1A mutants (Fig. 1, B, lanes 4–9, and C, lanes 3,4). These results are consistent with previous experiments performed with TFIID-depleted extracts and with partially purified factors, which showed that TBP cannot support the basal activity of consensus Inr elements (Kaufmann and Smale 1994; Martinez et al. 1994).

A simple explanation for the lack of Inr activity in the experiments described above is that the TFIID complex is missing from the reactions. To address this possibility, reconstitution experiments were performed with TFIIB, RAP30, RAP74, conventionally purified RNA polymerase II, and either a crude TFIID-containing fraction [phosphocellulose P11 D-fraction (1 M KCl eluate)] or immunopurified TFIID. With the crude TFIID fraction, strong Inr activity was detected, as indicated by the greatly enhanced strength of the promoter containing the wild-type Inr relative to the promoters containing the +3C or -1A mutant Inrs (Fig. 1B, lanes 13–15). In contrast, with immunopurified TFIID, very little Inr activity was observed, as the promoter containing the wild-type Inr was only slightly stronger than any of the three mutant promoters (Fig. 1, B, lanes 10–12, and C, lanes 5,6). Similar results were obtained when the reactions were performed with immunopurified RNA polymerase II, but the signals were much weaker (data not shown). These experiments strongly suggest that Inr activity requires proteins that are not required for TATA activity. Apparently, the essential proteins are present in the crude TFIID fraction but not in the immunopurified TFIID preparation.

Isolation of a protein fraction that imparts Inr activity

The above experiments suggest that TFIID-dependent Inr activity requires a factor that is not needed for TATA-directed transcription. To investigate this hypothesis, we set out to isolate the putative Inr-specific factor by fractionating HeLa cell extracts.

The fractionation studies described below greatly benefited from our ability to identify a protein fraction from HeLa cells that supports TATA-mediated transcription but not Inr activity (Fig. 2B, lanes 3,4). This fraction, which we call the IA⁻ (initiator activity minus) fraction, was isolated by sequential phosphocellulose, DEAE-Sephacel, and heparin-Sepharose chromatography (Reinberg and Roeder 1987; see Materials and methods; Fig. 2A). The IA⁻ fraction provides an abundant source of general transcription factors for analysis of column frac-

Figure 1. RNA polymerase II, TFIIB, RAP30, RAP74, and either TBP or immunoaffinity-purified TFIID are not sufficient for Inr activity. (A) The purified TFIID and TBP preparations were visualized by SDS-PAGE. Lanes 1 and 2 each contain 15 μ l of purified TFIID; and lane 3 contains 50 ng (determined by Bradford assay) of TBP. The epitope-tagged TBP (eTBP) within the TFIID complex migrates slightly slower than the 45-kD recombinant TBP. (B) In vitro transcription reactions were performed with supercoiled templates containing a TATA box and the wild-type TdT Inr (lanes 1, 4, 7, 10, 13), an Inr containing a mutation at +3 (lanes 2, 5, 8, 11, 14), or an Inr containing a mutation at -1 (lanes 3, 6, 9, 12, 15). RNA synthesis was measured directly with templates containing a G-less cassette, which yields a 180-nucleotide product (see Materials and methods). In vitro transcription assays were performed with HeLa nuclear extracts (lanes 1–3) or with RNA polymerase II, TFIIB, RAP30, RAP74, and either 10 ng of TBP (lanes 4–6), 40 ng of TBP (lanes 7–9), 5 μ l of TFIID (lanes 10–12), or 2 μ l of phosphocellulose D fraction (lanes 13–15). (C) In vitro transcription reactions were performed as described above but with promoters containing a TATA box and either the wild-type TdT Inr (lanes 1, 3, 5) or the Inr containing mutations at both +3 and -1 (lanes 2, 4, 6).

tions for the putative Inr-specific factor. Furthermore, it should be noted that the identification of a HeLa cell fraction that fails to support Inr activity strengthens the hypothesis that Inr activity requires an additional factor that is not needed for basal TATA-mediated transcription.

To begin to isolate the Inr-specific factor, fractions from the phosphocellulose and DEAE-Sephacel columns diagrammed in Figure 2A were added to the IA⁻ fraction. Inr activity was restored by addition of the 0.5 or 1.0 M KCl fractions from the phosphocellulose column (data not shown), or by addition of the 0.1 M fraction from the DEAE-Sephacel column (Fig. 2B, lanes 9, 10). The 0.25 M eluate from the DEAE-Sephacel column may also restore Inr activity, as transcription from the wild-type Inr was stronger than that from the mutant Inr when this fraction was added to the reactions (Fig. 2B, lanes 5, 6). However, the signal obtained with the wild-type Inr in

the presence of the 0.25 M fraction is not enhanced relative to the signal obtained with the IA⁻ fraction by itself. Thus, it is not clear whether the 0.25 M fraction contains a transcriptional repressor that only acts on the mutant promoter or an Inr stimulatory protein in combination with a general repressor of RNA polymerase II transcription.

With both the 0.1 and 0.25 M KCl fractions from the DEAE-Sephacel column, the ratios of wild-type to mutant promoter strengths were comparable to the ratios obtained with crude nuclear extracts or with the phosphocellulose C fraction (see Fig. 1). For subsequent experiments, we chose to focus on the DEAE 0.1 M KCl fraction rather than the 0.25 M KCl fraction because of the uncertainty regarding the nature of the activity in the 0.25 M fraction. Furthermore, previous studies had shown that most of the known general transcription factors are present primarily in the DEAE 0.25 M fraction

A **B**

Figure 2. DEAE-Sephacel fractions restore Inr activity to a protein preparation that supports TATA activity, but not Inr activity. (A) Fractionation scheme for HeLa nuclear extracts (see Materials and methods). (B) In vitro transcription assays were performed with plasmids containing a TATA box and the wild-type (odd-numbered lanes) or -1A + 3C mutant (even-numbered lanes) Inr element, with a concentrated phosphocellulose P11 C fraction (lanes 1,2), the IA⁻ fraction alone (lanes 3,4), or the IA⁻ fraction in combination with DEAE-Sephacel fractions (lanes 5–10). A control experiment was performed with the DEAE-Sephacel 0.1 M KCl eluate alone (lanes 13,14) or in combination with the IA⁻ fraction (lanes 11,12).

(Reinberg and Roeder 1987; White et al. 1992). Indeed, the DEAE 0.25 M fraction gave rise to the IA⁻ fraction that contains all of the general factors needed for TATA-mediated transcription (see Fig. 2A). In contrast, the DEAE 0.1 M fraction does not support basal TATA-mediated transcription (Fig. 2B, lanes 13,14). We will refer to the active Inr stimulatory components within this DEAE fraction as CIF.

The general transcription factors are not responsible for CIF activity

Several known proteins must be considered as potential candidates for CIF, including each of the general transcription factors. Even the general factors that are present in the IA⁻ fraction must be considered as candidates, as it is possible that Inr activity depends on high

concentrations of a specific general factor. To test whether Inr activity can be restored by the addition of excess TFIIB, RAP30, RAP74, TBP, TFIID, or RNA polymerase II, we attempted to complement the IA^- fraction with each of these proteins (Fig. 3). All of the proteins tested are known to be active because the same preparations were used for the reconstitution experiments shown in Figure 1. Furthermore, the TFIID used for this experiment supports Inr-mediated transcription when added to a heat-treated nuclear extract (data not shown). Interestingly, none of the proteins influenced Inr activity, but TFIIB and TBP stimulated transcription from both the wild-type and mutant promoters. Purified TFIIA, TFIIE, and TFIIH, obtained from other laboratories, were also added to the IA^- fraction, with no effect on Inr activity (data not shown). These results strongly suggest that the known general transcription factors are not solely responsible for the CIF activity detected in the DEAE 0.1 M KCl eluate.

CIF supports Inr activity in a TFIID-dependent manner

The above experiments demonstrate that the DEAE 0.1 M (CIF) fraction stimulates Inr activity when added to the IA^- fraction, which appears to contain all of the factors required for TATA-mediated transcription, including the TFIID complex. However, we have not yet determined whether the Inr stimulatory activity depends on the TFIID complex or is equally functional with TBP. To address this issue, the CIF fraction was tested in the presence of the purified and recombinant general transcription factors supplemented with either purified TFIID or TBP (Fig. 4). In the presence of purified TFIID, the CIF fraction stimulated Inr activity, as revealed by the enhanced signal obtained with the promoter containing the wild-type Inr (Fig. 4, lanes 5,6). In contrast, with TBP, equivalent transcription signals were observed with both the wild-type and mutant promoters (Fig. 4, lanes

3,4). The TBP used in this experiment appeared to direct basal TATA-mediated transcription more efficiently than did the purified TFIID (cf. lanes 4 and 6) but reduced concentrations of TBP also failed to reveal Inr activity (data not shown). These results demonstrate that the CIF fraction mediates Inr activity only in reactions containing the TFIID complex.

Partial purification of CIF activity

For further fractionation of the CIF activity, the DEAE-Sephacel 0.1 M fraction was subjected to sequential column chromatography with three additional resins to which the CIF activity bound (see Fig. 2A). CIF activity was eluted from each column with a linear salt gradient, and the fractions were assayed by complementation of the IA^- fraction. Active fractions were then pooled, dialyzed, and applied to the next column. The activity consistently eluted in a broad peak, and therefore, each step removed from the CIF preparation primarily those proteins that did not bind to the columns. Nevertheless, the results show that CIF activity could be efficiently bound to and recovered from multiple resins.

The three resins used for this partial purification procedure were phenyl-Sephacel, heparin-Sephacel, and a Mono-Q FPLC resin. Figure 5A shows the activities of equal volumes of the pooled fractions from each column. Strong CIF activity was retained through the phenyl-Sephacel and heparin-Sephacel columns, as the ratio of the signal obtained with the wild-type promoter relative to the mutant promoter increased from 4.2 to 8.2. As a result of the broad elution profiles, however, the specific activity was only moderately enhanced. A more extensive increase in specific activity was achieved with the Mono-Q FPLC resin, but the activity recovered was less concentrated, yielding a wild-type to mutant Inr ratio of only 3.4:1.

For further purification of the CIF activity, column

Figure 3. The IA^- fraction cannot be complemented by immunopurified TFIID, RNA polymerase II, TFIIB, TFIIF, or TBP. In vitro transcription assays were performed with nuclear extract (lanes 1,2) or with the IA^- fraction (lanes 3–18) supplemented with TFIID, RNA polymerase II, TFIIB, RAP30, and RAP74 as indicated (same amounts as in Fig. 1; see Materials and methods). Each combination was tested with plasmids containing the TATA box and either the wild-type (odd-numbered lanes) or mutant (+3C–1A; even-numbered lanes) Inr elements.

Figure 4. A CIF-containing fraction imparts Inr activity only in the presence of the TFIID complex. In vitro transcription assays were performed with RNA polymerase II, TFIIB, RAP30, and RAP74 (same amounts as in Fig. 1; see Materials and methods) in combination with a CIF-containing fraction (DEAE 0.1 fraction, 2 μ l) and either TBP (20 ng) (lanes 3,4) or immunopurified TFIID (4 μ l) (lanes 5,6). Each combination was tested with plasmids containing the TATA box and either the wild-type or mutant (+3C-1A) Inr elements as indicated. Control reactions were performed with HeLa nuclear extract (lanes 1,2) or with general factors and the DEAE 0.1 fraction in the absence of TBP and TFIID (lanes 7,8).

chromatography was performed with a DNA affinity resin containing multimerized oligonucleotides containing the TdT Inr sequence. No activity eluted from this column in the 0.1 or 0.5 M KCl fractions (data not shown). When low concentrations of the 0.25 M KCl fraction were added to the IA⁻ fraction, however, Inr activity was detected as was evident from the 4.2-fold enhanced activity of the promoter containing the wild-type Inr (Fig. 5B, lanes 5,6). (It should be noted that we have no evidence to suggest that the functional protein binds to the column in a sequence-specific manner. In fact, the elution of the activity with a relatively low salt concentration suggests that binding may not be sequence specific.) Interestingly, when higher concentrations of this fraction were added, the Inr activity for transcripts initiating at the expected nucleotide did not increase, but Inr-dependent transcripts were observed that were several nucleotides longer than the correctly initiated transcripts (Fig. 5B, lanes 7,8). Most likely, the longer transcripts are attributable to imprecise initiation at heterogeneous upstream sites, with the heterogeneous transcripts then cleaved by the RNase T1 that is added to the reactions. Because high concentrations of the cruder CIF fractions induce strong and highly specific Inr activity, those fractions most likely contain additional factors that contribute to CIF activity (see Discussion). Interestingly, a silver-stained protein gel revealed only one unique band in the 0.25 M KCl fraction (Fig. 5C, lane 5). This band migrated with a molecular mass of ~150 kD.

The affinity-purified CIF activity may be the mammalian homolog of Drosophila TAF_{II}150

The identification of a specific 150-kD band in the affinity purified CIF fractions was intriguing, given the considerable evidence suggesting that the *Drosophila* TAF_{II}150 protein binds to Inr elements and is responsible for Inr recognition (see introductory section and Discussion). A TAF_{II}150 homolog has not been detected in the purified mammalian TFIID preparations that bind to Inr elements and that support Inr-mediated transcription

(Zhou et al. 1992; Kaufmann and Smale 1993; Martinez et al. 1994), suggesting that it is either present at substoichiometric levels or only weakly associated with the TFIID complex and therefore missing from the purified preparations. Unfortunately, efforts to isolate the gene encoding the mammalian TAF_{II}150 homolog have been unsuccessful (C.P. Verrijzer and J. Kaufmann, unpubl.). In addition, antisera directed against the *Drosophila* TAF_{II}150 protein failed to detect a potential mammalian homolog in crude HeLa nuclear extracts (Fig. 6B, lane 1). The only evidence to support the presence of a human TAF_{II}150 homolog was provided by Jacq et al. (1994), who identified a 150-kD band in a TFIID-like complex that lacks the TAF_{II}30 protein.

To gain insight into the possibility that a TAF_{II}150 homolog is a component of CIF, immunoblot experiments were performed with column fractions containing CIF activity. Importantly, the polyclonal antibodies used for these experiments were raised against recombinant *Drosophila* TAF_{II}150 and were affinity purified on a column containing purified, recombinant TAF_{II}150 protein. The CIF-containing column fractions used for the initial immunoblot analysis were recovered from a Sephacryl S-300 gel filtration column. With this resin, peak CIF activity elutes with an apparent molecular mass of ~200-kD, which corresponds to fractions 10 and 11 for the column whose activity is shown in Figure 6A. Interestingly, the immunoblot analysis revealed a 150-kD band that cross-reacts with the affinity-purified *Drosophila* TAF_{II}150 antibodies (Fig. 6B, lanes 4-10). This cross-reactive band eluted from the S-300 column in the same fractions as the functional CIF activity, being most abundant in fractions 10 and 11 (lanes 6,7).

A second immunoblot experiment, shown in Figure 6C, confirms that CIF activity copurifies with the 150-kD protein that cross-reacts with affinity-purified *Drosophila* TAF_{II}150 antibodies. For this experiment, CIF activity was fractionated through six columns by use of the complementation assay with the IA⁻ fraction. This fractionation procedure was somewhat different from that diagrammed in Figure 2A: Following the phosphocellulose

Figure 5. A 150-kD polypeptide coelutes with CIF activity. (A) In vitro transcription reactions were performed with HeLa nuclear extract (lanes 1,2), the IA⁻ fraction alone (lanes 3,4), and the IA⁻ fraction in combination with the pooled CIF fractions after a DEAE-Sepharose anion-exchange column [2 μl (1.6 mg/ml), lanes 5,6], a phenyl-Sepharose column [2 μl (0.25 mg/ml), lanes 7,8], a heparin-Sepharose column [2 μl (0.2 mg/ml), lanes 9,10] and a Mono Q FPLC column [2 μl (0.03 mg/ml), lanes 11,12]. Each combination was tested with plasmids containing the TATA box and either a wild-type (odd-numbered lanes) or mutant (+3C-1A; even-numbered lanes) Inr. The ratios of the signals obtained with the two promoters are shown at the bottom, as are the protein concentrations of the pool of active fractions from each column. (B) In vitro transcription reactions were performed with HeLa nuclear extracts (lanes 1,2), the IA⁻ fraction alone (lanes 3,4), and the IA⁻ fraction in combination with 2 μl (lanes 5,6) or 4 μl (lanes 7,8) of the 0.25 M eluate from the DNA affinity column (see Material and methods). Arrows indicate the two transcription initiation sites observed. (C) The loading material (lane 1), flowthrough (lane 3), and the 0.1, 0.25, and 0.5 M eluates (lanes 4–6) from the DNA-affinity column were analyzed on an 8% SDS-polyacrylamide gel. Twenty microliters of each sample was loaded onto the gel. Proteins were detected by silver staining. A 150-kD protein, which is enriched in the 0.25 M eluate, is indicated by an arrow. Molecular mass standards were also included (lane 2), with sizes indicated to the left.

and DEAE-Sepharose columns, the active CIF fractions were sequentially applied to heparin-Sepharose,

Sephacryl S-300, phenyl-Sepharose, and heparin-agarose columns. The active fractions that resulted from this

purification procedure restored strong CIF activity when added to the IA^- fraction (Fig. 6C, lanes 7–10). The immunoblot analysis that is shown (Fig. 6C, lanes 1–6) was performed with 15 μ l each of the HeLa extract (lane 2), the phosphocellulose C fraction (lane 3), and the active eluates from the final two columns in the purification procedure (lanes 4,5). The only bands detectable in the extract and phosphocellulose fraction (lanes 1,2) were those that react with the secondary antibody alone (data not shown; see Fig. 6B, right). Following the fifth and sixth columns, however, the 150-kD band that cross-reacts with the *Drosophila* TAF_{II}150 antibodies became greatly enriched and easily detectable (lanes 4,5). A very weak band was also detected by immunoblot analysis of the active 0.25 M KCl eluate from a DNA affinity column (data not shown; see Fig. 5C).

The copurification of the 150-kD immunoreactive band with CIF activity suggests that the human homolog of *Drosophila* TAF_{II}150 is a component of CIF. To analyze the role of TAF_{II}150 in CIF function directly, we added purified recombinant *Drosophila* TAF_{II}150 to the HeLa-derived IA^- fraction. The result obtained was strikingly similar to that obtained with the affinity-purified CIF fractions (Fig. 7). With low concentrations of recombinant TAF_{II}150, weak Inr activity was consistently detected, as indicated by the 2.6-fold higher signal obtained with the wild-type promoter relative to the mutant promoter (Fig. 7, lanes 5,6). Interestingly, with higher concentrations of *Drosophila* TAF_{II}150 (lanes 7,8), the longer transcripts appeared that were detected previously with the affinity-purified CIF fraction. The striking similarity between the activities of the affinity-purified human CIF component and the recombinant TAF_{II}150 strongly suggest that a mammalian TAF_{II}150 homolog is partially responsible for CIF activity. The inability of either the human 150-kD protein or the *Drosophila* TAF_{II}150 to activate strong Inr activity from the correct start site suggests, however, that complete CIF activity requires additional proteins that are present in the partially purified CIF preparations.

Discussion

The TATA box and Inr are core promoter elements capable of directing accurate basal transcription initiation by RNA polymerase II. These elements also function in concert with one another to direct transcription initiation at elevated levels (Smale and Baltimore 1989; Smale et al. 1990). Both elements appear to function similarly during transcription initiation by providing a recognition site for TFIID (Kaufmann and Smale 1994; Purnell et al. 1994; Verrijzer et al. 1994, 1995). Several additional characteristics of the transcription initiation process appear to be similar for promoters that contain either a TATA box or an Inr (Smale 1994). On the basis of these observations, we and others had postulated that TFIID-dependent Inr activity may depend solely on the general transcription factors that are involved in TATA-mediated transcription (Pugh and Tjian 1991; Wiley et al. 1992; Aso et al. 1994; Martinez et al. 1994; Smale 1994).

The data outlined in this report demonstrate that Inr activity from a promoter that contains both a TATA box and an Inr requires a factor, CIF, that is not needed for basal TATA-mediated transcription. The CIF factor, which has been partially purified, functions only in the presence of the TFIID complex and appears to contain multiple components. Functional and immunological experiments indicate that one of the CIF components is the mammalian homolog of *Drosophila* TAF_{II}150 (Verrijzer et al. 1994), which is not tightly associated with mammalian TFIID. We have not yet determined whether the CIF components are required for Inr activity from a promoter that lacks a TATA box, but we suspect that the protein requirements for Inr activity from a TATA-less promoter will be similar to, if not more complicated than, the requirements for Inr activity in the presence of a TATA box.

The TdT Inr (TCA₊TTCT) used in this study matches the loose consensus sequence for Inr activity in mammalian cells (PyPyA₊NT/APyPy). This consensus was defined by functional analysis of random and mutant Inr elements (Javahery et al. 1994) and is similar to a start-site consensus defined through a sequence comparison of 500 genes transcribed by RNA polymerase II (Bucher 1990). Therefore, we suspect that CIF will be required for the activity of many, if not all, Inr elements matching this consensus.

The partial restoration of CIF activity by recombinant *Drosophila* TAF_{II}150 and by the antigenically related human 150-kD protein confirms the importance of TAF_{II}150 for Inr function. The first experiments suggesting a role for TAF_{II}150 in Inr function were provided by Verrijzer et al. (1994, 1995), who found that recombinant *Drosophila* TAF_{II}150 bound to Inr elements and that a TBP-TAF_{II}250-TAF_{II}150 complex was competent for supporting Inr activity when added to partially purified general transcription factors from *Drosophila*. More recently, *Drosophila* TAF_{II}150 was found to be functionally important for distinguishing between a promoter containing a consensus Inr and a closely linked promoter lacking an Inr (Hansen and Tjian 1995). The role of a TAF_{II}150 homolog in the function of mammalian Inr elements has remained uncertain, however, as a TAF_{II}150 homolog has not been detected in the highly purified mammalian TFIID preparations that support Inr activity (Zhou et al. 1992; Chiang et al. 1993; Kaufmann and Smale 1994; Martinez et al. 1994). Cloning of the mammalian TAF_{II}150 gene with heterologous probes has also not yet succeeded (C.P. Verrijzer and J. Kaufmann, unpubl.).

The appearance of aberrantly initiated transcripts following addition of high concentrations of *Drosophila* TAF_{II}150 and the affinity purified CIF fraction suggests that additional proteins present in the partially purified CIF fractions are essential for full Inr activity. The size of the aberrant band suggests that it most likely results from initiation at multiple upstream locations, with the heterogeneous transcripts cleaved by the RNase T1 that is added to the completed reactions (RNase T1 cleaves adjacent to guanosine residues that are not encoded

Figure 6. (See facing page for legend.)

Figure 7. Recombinant *Drosophila* TAF_{II}150 and the 150-kD CIF component exhibit similar activities in a complementation assay. In vitro transcription reactions were performed with HeLa nuclear extract (lanes 1,2), the IA⁻ fraction alone (lanes 3,4), and the IA⁻ fraction in combination with increasing amounts (2 and 4 μ l) of either recombinant *Drosophila* TAF_{II}150 (lanes 5–8) or of the 0.25 M DNA affinity eluate (lanes 9–12; see also Fig. 5B). The plasmids used contained the TATA box and either the wild-type (odd-numbered lanes) or mutant (even-numbered lanes) Inr elements. Arrows indicate the two transcription initiation sites observed.

within the G-less cassette). It is important to note that in general, these aberrant transcripts remain Inr dependent; thus, although they may be heterogenous, we suspect that they initiate in the vicinity of the Inr. The apparent loss of sequence specificity that is observed with high protein concentrations is interesting to consider in light of the fact that TAF_{II}150 binds to DNA with relatively limited sequence specificity (Verrijzer et al. 1994, 1995), particularly with high concentrations of protein (C.P. Verrijzer, unpubl.). Thus, although the aberrantly initiated transcripts currently cannot be explained, they are likely to result from imprecise DNA binding by TAF_{II}150. The additional components of CIF may therefore act by increasing the precision of the TAF_{II}150/DNA interaction.

The results presented here strongly suggest that a human TAF_{II}150 homolog exists but that this protein is not tightly associated with the TFIID complex. It remains to be established whether the human TAF_{II}150 protein assembles into preinitiation complexes on core promoters that contain only a TATA box or whether it becomes

part of the preinitiation complex only when the core promoter contains an Inr. It is important to note that highly purified human TFIID has been shown to bind to Inr elements, with the interaction dependent on the nucleotides needed for Inr function (Kaufmann and Smale 1994). It remains possible that this interaction is mediated by a small amount of the TAF_{II}150 homolog, which may be tightly associated with the purified TFIID preparation. Alternatively, another protein within the TFIID complex (e.g., TAF_{II}250) may contribute to Inr recognition. We favor this latter possibility, as the *Drosophila* TAF_{II}150–Inr interaction is not as sensitive to point mutations within the Inr as predicted by the functional studies of Inr elements (C.P. Verrijzer, unpubl.). Thus, a plausible model is that TAF_{II}150 is essential for Inr function and binds to Inr elements with limited sequence specificity, with a second TAF providing essential, sequence-specific contacts with the Inr.

The absence of a tight interaction between the TFIID complex and the human 150-kD protein is reminiscent of the properties of the TFIID–TFIIA interaction. Both

Figure 6. CIF activity coelutes with a 150-kD polypeptide that cross-reacts with an antiserum directed against *Drosophila* TAF_{II}150. (A) In vitro transcription reactions were performed with the IA⁻ fraction in combination with different fractions from a Sephacryl S-300 gel filtration column with promoters containing the wild-type or mutant Inr elements (lanes 7–18). Control reactions were performed with nuclear extract (lanes 1,2), the IA⁻ fraction alone (lanes 3,4), and the IA⁻ fraction in combination with the loading material for the gel filtration column (lanes 5,6). (B) Immunoblot assays were performed with HeLa nuclear extract (lane 1), the IA⁻ protein fraction (lane 2), a *Drosophila* nuclear extract (lane 3), or gel filtration fractions 8–14 (lanes 4–17), following SDS-PAGE with an 8% gel. The affinity-purified *Drosophila* TAF_{II}150 antibodies were used for the immunoblots shown in lanes 1–10. A control immunoblot with only the secondary antibody (biotinylated anti-rabbit immunoglobulin) is shown in lanes 11–17. The *Drosophila* TAF_{II}150 protein and the cross-reacting human protein are indicated by arrows. The sizes of the immunoreactive polypeptides were estimated from the markers as indicated. (C) CIF activity was fractionated through six columns (see Results). (Left) 15 μ l each of crude HeLa extract (lane 2), phosphocellulose C fraction (lane 3), and active fractions following the phenyl–Sephacryl (lane 4) and heparin–agarose (lane 5) columns were analyzed by immunoblot following SDS-PAGE on a 6% gel. The immunoblot analysis was performed with the affinity-purified *Drosophila* TAF_{II}150 antibodies. The arrow indicates the 150-kD band that cross-reacts with the antibodies. The strong bands at the top and bottom are attributable to reactivity with the secondary antibody (data not shown; see B). Prestained markers were loaded in lanes 1 and 6, with the 200-kD marker interacting with the secondary antibody. (Right) The activity of the six-column-purified CIF fraction (2 μ l) was measured by complementation of the IA⁻ fraction, as described in the legends to Figs. 2B and 5A.

TFIIA and TAF_{II}150 are tightly associated with the TFIID complex in *Drosophila* cells but not in human cells (Yokomori et al. 1993). It may be noteworthy that TFIIA and TAF_{II}150 may have related functions, as TFIIA appears to contribute to the TFIID–TATA interactions, whereas TAF_{II}150 contributes to the TFIID–Inr interaction. Possibly, the decreased affinities between TFIID and both TFIIA and TAF_{II}150 provided an advantage during mammalian evolution.

For the identities of the other components of CIF to be determined, they will need to be purified to homogeneity and their genes cloned. These additional components carry out dual roles of increasing Inr activity as well as decreasing the imprecise initiation. Because we have been unable to restore Inr activity with general transcription factors, the CIF components may be novel. In addition, it is unlikely that the reported Inr-binding proteins, TFII-I, USF, or YY1 are components of CIF because these proteins function in the presence of TBP (Roy et al. 1991; Seto et al. 1991; Du et al. 1993) or in the complete absence of TBP or TFIID (Usheva and Shenk 1994), whereas TFIID is essential for CIF activity.

Finally, the identification of a factor that is needed for Inr activity, but not for TATA activity, may provide insight into the question of why some promoters contain TATA boxes, and others contain Inr elements. If, as suggested previously (see introductory section), TATA- and Inr-mediated transcription reactions were functionally and mechanistically very similar, it would be difficult to explain why core promoters evolved to contain heterogeneous structures, other than by random chance. The requirement for additional factors suggests that transcriptional activation through an Inr-containing promoter may depend on CIF-dependent protein–protein interactions. Recent experiments (Emami et al. 1995; Garraway et al., 1996) have revealed that some activators, including the glutamine-rich domain of Sp1 and an activator of the murine TdT promoter, can function efficiently only if the core promoter contains an Inr. The identification of CIF may lead to an understanding of the Inr preference of these activators; the activators may specifically interact with components of CIF and therefore stimulate transcription only when the promoter contains an Inr. Further characterization of CIF will reveal its relevance to these Inr-dependent activation events.

Materials and methods

Purification of basal transcription factors

The epitope-tagged TFIID complex was isolated from LTR α 3 cells by immunoaffinity purification as described (Zhou et al. 1992). RNA polymerase IIA was immunoaffinity purified by the method of Thompson et al. (1990) with the monoclonal antibody 8WG16 [a kind gift of R.R. Burgess (University of Wisconsin, Madison), and A. Berk (University of California, Los Angeles (UCLA))] coupled to protein A–Sepharose. A concentrated P11 C fraction from HeLa nuclear extracts was the source of the immunoaffinity-purified polymerase. For partial purification of RNA polymerase II from HeLa cells, the 1 M KCl eluate from the DEAE–Sephacel column (see Fig. 3A) was dialyzed against

buffer A (20 mM HEPES at pH 7.9, 1 mM EDTA, 3 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20% glycerol) with 0.1 M KCl (Zheng et al. 1987). Recombinant TBP and TFIIB were prepared from *E. coli* as described previously (Peterson et al. 1990; Ha et al. 1991; Zhou et al. 1992). Purified, recombinant RAP30 and RAP74 were kindly provided by R. Koop, K. Ahrens, and A. Berk (UCLA).

Fractionation of CIF

HeLa nuclear extracts (200 ml, 20 mg/ml) were prepared as described previously (Dignam et al. 1983) and applied to a 100-ml phosphocellulose P11 column (Whatman). The phosphocellulose P11 C fraction (step elution with 0.3 to 0.5 M KCl in buffer A) was used for subsequent purification steps. The P11C fraction was concentrated by ammonium-sulfate precipitation (30%) and dialyzed against buffer A containing 0.1 M KCl. This fraction (75 mg) was applied to a DEAE–Sephacel (Pharmacia) column at ~5 mg of total protein per milliliter of resin, and the column was subsequently washed with buffer A containing 0.1 M KCl, 0.25 M KCl, and 1 M KCl (three column volumes each). The fractions were pooled and dialyzed against buffer A with 0.1 M KCl. The 0.25 M KCl eluate of the DEAE–Sephacel column was applied to a 15-ml heparin–Sepharose column (see Fig. 2A). Proteins were eluted with a linear gradient (30 ml) from 0.1–1 M KCl in buffer A. In vitro transcription assays revealed the IA[−] protein fraction (4 mg/ml), which supported TATA-mediated transcription, but not Inr activity.

For the partial purification of CIF, the 0.1 M KCl flowthrough fraction of the DEAE–Sephacel column (80 ml, 1.6 mg/ml) (adjusted to 1 M KCl) was applied to a phenyl–Sepharose column (20 ml) and eluted with a linear KCl gradient (40 ml; 1.0–0.1 M). The CIF-containing fractions were pooled and dialyzed against buffer A containing 0.1 M KCl. This sample (30 ml, 0.25 mg/ml) was then applied to a heparin–Sepharose column (15 ml) and eluted with a linear KCl gradient as described above. CIF-containing heparin–Sepharose fractions were pooled and dialyzed against buffer A (0.1 M KCl). The dialyzed heparin–Sepharose pool (7 ml, 0.2 mg/ml) was applied to a Mono Q FPLC column (Pharmacia, 2 ml). After elution with a 30-ml linear gradient (0.1–1 M KCl in buffer A), the CIF-containing fractions were pooled and diluted to 0.1 M KCl with buffer A. The diluted Mono Q pool (2 ml, 0.03 mg/ml) was loaded onto a 2-ml sequence-specific DNA-affinity column (Kadonaga and Tijan 1986), which contained multimers of the oligonucleotide 5'-ACATCAGAGCCCTCATTCTGGAGA-3' and its complementary sequence. The column was washed with two column volumes of buffer A containing 0.1 M KCl. Bound proteins were eluted sequentially with two column volumes of buffer A containing 0.25 M KCl and buffer A containing 0.5 M KCl. Protein fractions were tested for CIF activity, and polypeptides were visualized by SDS-PAGE (8%), followed by silver staining.

For the experiment shown in Figure 6C, the chromatography conditions employed were similar to those described above, except the columns were arranged in the order described in the Results.

Western blot analysis

Protein fractions used in the immunoblot experiment were derived from an 80-ml gel filtration column (Sephacryl S-300, Pharmacia). The loading material was 2 ml of the DEAE 0.1 M KCl eluate (see above). Fractions (3 ml) were collected and 30- μ l aliquots were boiled for 5 min in Laemmli sample buffer, resolved by 8% SDS-PAGE, and transferred to nitrocellulose membrane. The blot was blocked for 2 hr with 5% fat-free dried

milk in 50 mM Tris-HCl (pH 7.4) and 0.5 M NaCl. Then, the membrane was incubated for 2 hr each with a 1:500 dilution of a polyclonal antibody directed against *Drosophila* TAF_{II}150 (Verrijzer et al. 1994), with biotinylated anti-rabbit immunoglobulin (BioGenex), and with peroxidase-conjugated streptavidin (BioGenex) in an antibody dilution buffer (50 mM Tris-HCl at pH 7.4 and 0.5 M NaCl). Between each incubation, the membrane was washed three times with wash buffer (50 mM Tris-HCl at pH 7.4, 0.5 M NaCl, and 0.2% Tween 20). After the last incubation, the membrane was washed for 20 min and developed with an ECL kit (Amersham). The polypeptide that cross-reacts with the *Drosophila* TAF_{II}150 antibody was visualized on Kodak XAR5 film.

Plasmid DNAs

Plasmid DNAs containing TATA and Inr elements upstream of a 180-bp G-less cassette were constructed by use of a PCR protocol. The upstream PCR primers contained a *Sac*I site and the different Inr sequences (TdT wild-type: 5'-AAATGGAGCTC-CACACCAAATCCCTCATTCTACCCTTCCTC-3'), with the sequences immediately flanking the Inr derived from the IgH promoter and from the beginning of the G-less cassette. The downstream primer contained a *Bam*H I site (5'-GGAAG-GATCCTGGAGGGGATATGGAAGGGA-3') and was complementary to a sequence of the standard G-less sequence [pMu(-47)-(G)-I; Parvin and Sharp 1991]. PCR was performed with *Taq* polymerase (Perkin Elmer) by use of conditions recommended by the manufacturer. The PCR products were cleaved with *Sac*I and *Bam*HI and inserted into pSP72 (Promega). A double-stranded oligonucleotide containing a consensus TATA-box (5'-AATTCGAGGGCTATAAATGGGAGCT-3') was then inserted into the *Eco*RI and *Sac*I sites of these plasmids, yielding TGLwt, TGL+3C, TGL-1A, and TGL+3C-1A. All plasmid DNAs were amplified in *E. coli* and purified by a standard plasmid preparation protocol (Qiagen).

In vitro transcription assays

In vitro transcription reactions were performed with the templates containing the G-less cassette as described (Goodrich and Tjian 1994). The final reaction volume was 35 μ l in GL-buffer (10 mM Tris-HCl at pH 7.9, 10 mM HEPES at pH 7.9, 10% glycerol, 1 mM DTT, 4 mM MgCl₂, 50 mM KCl, 10 mM ammonium sulfate, 100 μ g/ml of BSA). For the complementation assay, 2 μ l of the IA⁻ fraction (4 mg/ml) was preincubated in the presence of DNA template with 1–4 μ l of the CIF activity-containing fractions. Proteins used in the minimal reconstitution system were 20 ng of TBP, 20 ng of TFIIB, 30 ng of RAP30, 15 ng of RAP74, 3–9 μ l of immunopurified TFIID, 4 μ l of RNA polymerase IIA, or 1 μ l partially purified RNA polymerase II. The supercoiled DNA template concentration was 1–2 nM. Purified transcription factors were preincubated with DNA for 20 min at 30°C, followed by addition of rNTPs to yield the following final concentrations: 500 μ M ATP, 500 μ M CTP, and 30 μ M [α -³²P]UTP. The reactions did not contain 3'-O-methyl GTP. Reactions were incubated for 40 min at 30°C. RNase T1 (20 units; Boehringer Mannheim) was then added, followed by a 20 min-incubation at 30°C. Reactions were stopped by addition of 100 μ l of stop mix (20 mM EDTA, 1% SDS, 200 mM NaCl, 0.25 mg/ml of tRNA). The resulting aqueous solutions were extracted with a phenol/chloroform mixture, and the RNA was precipitated with ethanol. ³²P-labeled RNA products were resolved on an 8% polyacrylamide-urea gel and visualized by autoradiography. Signals were quantitated by PhosphorImager

analysis (Molecular Dynamics, Inc.) or by densitometry (SciScan 5000, U.S. Biochemical).

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