The Initiator Core Promoter Element Antagonizes Repression of TATA-directed Transcription by Negative Cofactor NC2

Barbora Malecová, Petra Gross, Michael Boyer-Guittaut, Sevil Yavuz, and Thomas Oelgeschläger
From the Transcription Laboratory, Marie Curie Research Institute, The Chart, Oxted, Surrey RH8 0TL, United Kingdom

Core promoter regions of protein-coding genes in metazoan genomes are structurally highly diverse and can contain several distinct core promoter elements, which direct accurate transcription initiation and determine basal promoter strength. Diversity in core promoter structure is an important aspect of transcription regulation in metazoans as it provides a basis for gene-selective function of activators and repressors. The basal activity of TATA box-containing promoters is dramatically enhanced by the initiator element (INR), which can function in concert with the TATA box in a synergistic manner. Here we report that a functional INR provides resistance to NC2 (Dr1/DRAP1), a general repressor of TATA promoters. INR-mediated resistance to NC2 is established during transcription initiation complex assembly and requires TBP-associated factors (TAFs) and TAF- and INR-dependent cofactor activity. Remarkably, the INR appears to stimulate TATA-dependent transcription similar to activators by strongly enhancing recruitment of TFIIA and TFIIIB and, at the same time, by compromising NC2 binding.

Accurate initiation of mRNA synthesis in eukaryotic cells requires assembly of RNA polymerase II (RNAP II) and general transcription factors (GTFs, TFIIA, -B, -D, -E, -F, and -H) into an initiation-competent nucleoprotein complex (preinitiation complex, PIC) at the core promoter. The core promoter encompasses a DNA region of ~80 bp from position −40 to +40 relative to the transcription start site (+1) and can be operationally defined as the minimal stretch of DNA that is sufficient to direct basal (activator-indepen-
dent) levels of accurate transcription initiation in vitro (1, 2). Metazoan core promoters can contain several distinct core promoter sequence elements, which direct functional PIC assembly and transcription start site selection and which determine basal promoter strength (1, 2).

Core promoter elements identified to date include the TATA box (TATA), the initiator element (INR), TFIIB recognition elements (BREs), the downstream promoter element (DPE), the motif-ten element (MTE), and the downstream core element (DCE) (1, 2). Computational analyses of metazoan genomes have revealed remarkable diversity in RNAP II core promoter architecture. Each of the core promoter elements identified so far is found in only a subset of core promoters, and there appears to be no core promoter element that is universally required for gene activity (1–6).

A plethora of biochemical studies, studies in cultured human cells and genetic studies in Drosophila have provided compelling evidence that the structural diversity of core promoters is an important aspect of gene regulation in metazoans. Core promoter structure has been shown to affect the amplitude by which genes respond to various activators and repressors and appears to play a crucial role in the specific communication between enhancers and their target genes (1, 2, 7–10).

So far, TATA and INR are the only core promoter elements known to be sufficient to direct accurate RNAP II transcription initiation in the absence of other core promoter elements. TATA-directed RNAP II transcription has been studied in great detail and is well understood. At TATA-containing promoters, PIC assembly is initiated by stable binding of the TATA-binding protein (TBP) subunit of TFIID to the TATA DNA sequence, which creates a platform for the recruitment of the remaining GTFs and RNAP II (11–14). At complex core promoters, TATA box function is modulated by additional core promoter elements such as BREs and the INR. BREs can be found immediately up- (BREu) and/or downstream (BREd) of promoter elements such as BREs and the INR. BREs can be found immediately up- (BREu) and/or downstream (BREd) of the TATA box sequence and can either stimulate or repress TATA-directed transcription (15–17). The INR encompasses the transcription initiation site and was originally identified as a discrete core promoter element essential for activity of the TATA-less murine terminal deoxynucleotidyl transferase (mTdT) gene promoter (18). TATA and INR can function cooperatively when separated by 25–30 bp (19), and TATA/INR cooperativity dramatically increases basal promoter activity compared with the presence of either TATA or INR alone (18, 20–22).

Up to date the vast majority of studies into the basic mechanisms underlying RNAP II transcription initiation were carried out with TATA-containing model promoters and little is...
INR Function Counteracts NC2 Repression

known about the molecular mechanisms underlying INR-directed transcription initiation. Bioinformatics analyses suggest that only a minority (<20%) of RNAP II promoters in the human genome contain a TATA element. In contrast, about half of the RNAP II promoters were found to contain an INR consensus sequence (4–6). Importantly, the basal RNAP II transcription machinery, which has been biochemically defined as the minimal set of protein factors necessary and sufficient to direct basal levels of accurate RNAP II transcription initiation at TATA-containing model promoters (TBP, TFIIA, E, F, H, RNAP II; Roeder (14)) is not sufficient to support INR-directed transcription initiation (23). INR-dependent TATA-less transcription as well as stimulation of TATA-dependent transcription by the INR requires in addition TAF subunits within TFIIA, TFIIA, and so-called TAF- and INR-dependent cofactor activity (TICs) (21–25). The identity of the peptides responsible for TIC activity is still not known. So far, reconstitution of INR-mediated transcription initiation with purified factors has not been achieved and INR function can only be studied in crude nuclear extracts (NE) or in reconstituted systems supplemented with partially purified TIC activity (2, 23).

In nuclear extracts, which provide a complement of nuclear factors at ratios close to the physiological situation, basal promoter strength is determined by a complex interplay between positive and negative cofactors that affect the activity of the basal RNAP II transcription machinery (26, 27). Among these, negative cofactor 2 (NC2; aka Dr1/DRAP1) has been well characterized as a potent repressor of basal transcription from TATA-containing promoters. NC2 is a heterodimer composed of NC2α and NC2β subunits, which interact through H2A/H2B-type histone-fold motifs. NC2 can repress basal promoter activity by binding to the TBP/TATA complex intermediate, which prevents recruitment of TFIIA and TFIIIB and, hence, PIC assembly (26, 28–33).

Here we show that the presence of a functional INR renders TATA-containing promoters resistant to NC2. Remarkably, stimulation of TATA-dependent transcription by the INR resembles an activator-dependent mechanism: INR stimulation strongly enhances recruitment of TFIIA and TFIIIB and, at the same time, inhibits NC2 binding during PIC assembly. These findings suggest that activators and the INR element stimulate basal promoter activity through similar or related mechanisms.

**Experimental Procedures**

**Promoter Constructs**—All promoter constructs used in this study are pGEM7zf (Promega) derivatives. pECHIV-1(−111/−80) and pTOHIV(−33/−80) contain HIV-1LTR promoter sequences from −111 to +80 and from −33 to +80, respectively (34). pPGTdT(TATA+/+59) contains within an ApaI/BamHI insert mTdT core promoter sequences from −41 to +59 with a consensus TATA box at position −30 (22) in front of 5 GAL4 binding sites. pPGTdT(TATADA1/NR+/+59) is a derivative of pPGTdT(TATA+/+59) containing six point mutations which eliminate INR function without affecting start site selection (22). pTOGHSHP(−49/+38) contains the human HP70 core promoter from −49 to +38 (35). pPGG5HSP(TdTINR) is a derivative of pTOGHSHP(−49/+38) in which hHS70 core promoter sequences from −4 to +5 were substituted by the corresponding mTdT promoter sequence containing the INR element. “L” variants (−L, Fig. 1) of mTdT and hHSP70 promoter constructs contain additional 26 bp of pGEM7zf polylinker sequence downstream of the core promoter region, which do not affect core promoter activity and which allow to analyze transcripts originating from different core promoter variants in parallel by primer extension using the same radioactive primer.

**Antibodies**—To generate antibodies for NC2α and NC2β, rabbits were immunized with peptides SAPDEEDEEDYDS (amino acid residues 193–205 in human NC2α) and SAS-NAGSSQDEEEDDD1 (amino acid residues 159–176 in human NC2β), respectively. Antibodies specific for the NC2α and NC2β epitopes were affinity-purified from selected rabbit antisera. Rabbit polyclonal anti-TFIIA (C-18; Santa Cruz Biotechnology) and mouse monoclonal anti-RNAP II CTD antibody (8WG16; Covance Research Products) are commercially available. Rabbit polyclonal antibodies for human TBP, TAF5, TFIIEα, TFIIAα/β, TFIIH p62, and human Srb7 (MED21) were kindly provided by R. G. Roeder (Rockefeller University). J. Kaufmann (Silence Therapeutics) provided rabbit polyclonal antibody for human TAF2 and A. Hoffmann (University of California, San Diego) rabbit polyclonal antibody for human TAF12.

**Nuclear Extracts and Proteins**—Extracts and proteins were stored in BC buffer (20 mM Tris-HCl, pH 7.9 at 4 °C, 0.2 mM EDTA, 10 mM β-mercaptoethanol, 20% glycerol) containing 100 mM KCl. NE were prepared as described (36). NE lacking NC2 (NE[ΔNC2]) was obtained by chromatography over protein A-Sepharose CL4B (Amersham Biosciences) resin covalently cross-linked to rabbit polyclonal antibody for human NC2α in BC buffer containing 500 mM KCl and 0.1% IGEPAL-CA-630 (Sigma). Depletion of TBP and TFIIA TAFs from HeLa NE or NE[ΔNC2] to generate NE lacking TFIIID (NE[ΔD] or NE[ΔΔNC2]) was carried out as described (35). The purification of GTFs and RNAP II for the reconstituted in vitro transcription is described in the supplemental information.

**Purification of Recombinant Human NC2 Complex (6His:NC2α/fNC2β)**—Human 6His-tagged NC2α and FLAG epitope:tagged NC2β subunits were co-expressed in Escherichia coli (see supplemental information) and rNC2 complex was purified by sequential affinity purification on Ni-NTA agarose (Qiagen) in BC-buffer containing 500 mM KCl and on anti-FLAG (M2) antibody agarose (Sigma) in BC-buffer containing 250 mM KCl and 0.1% IGEPAL-CA-630 (Sigma).

**In Vitro Transcription and Primer Extension Analysis**—In vitro transcription and primer extension analysis was carried out as described previously (34).

**Template Recruitment Assays**—DNA fragments containing promoter sequences of interest (HIV-1 core: 334 bp, transcription start site (TSS) at position 228; HIV1-activated: 373 bp, TSS at position 226, mTdT-TATA and mTdT-TATA/INR: 424 bp, TSS at position 222) with a 5′-Biotin link at the downstream end were generated by PCR, gel-purified, and immobilized on streptavidin-coated magnetic beads (Dynabeads M-280, Dynal) according to the manufacturer’s instructions. To assemble PICs, 200 fmol of immobilized promoter templates were incubated with HeLa nuclear extract protein and/or purified factors.
Depletion of NC2 from human nuclear extracts selectively enhances transcription form TATA-containing core promoters lacking an INR element. A, mTdT and B, hsHSP70 core promoter variants containing only a TATA element (TATA) or TATA and INR elements (TATA/INR) were transcribed in untreated HeLa NE or HeLa nuclear extract depleted of NC2 (NE[ΔNC2]). L variants of TATA promoter constructs contain an additional 26-bp polylinker sequence downstream of the core promoter region, which does not affect core promoter activity and allows the analysis of transcripts originating from TATA and TATA/INR variants in parallel by primer extension using the same radioactive primer. Transcription reactions (20 μl) were carried out at 30 °C for 1 h with the NE protein concentrations indicated. Transcripts from TATA and TATA/INR promoter variants were analyzed in parallel by primer extension using the same radiolabeled primer. Primer extension products were resolved by 6% denaturing PAGE and quantified by PhosphorImager analysis. C, immunoblot analysis of untreated NE (HeLa NE) and NE depleted of NC2 (NE[ΔNC2]). RNAP II was probed with antibody N-20, which recognizes an epitope at the N terminus of the largest subunit RPB1 and which reacts equally with CTD hyper-(IIO) and hypophosphorylated (IIA) RPB1 forms.

**FIGURE 1.** Depletion of NC2 from human nuclear extracts selectively enhances transcription form TATA-containing core promoters lacking an INR element. A, mTdT and B, hsHSP70 core promoter variants containing only a TATA element (TATA) or TATA and INR elements (TATA/INR) were transcribed in untreated HeLa NE or HeLa nuclear extract depleted of NC2 (NE[ΔNC2]). L variants of TATA promoter constructs contain an additional 26-bp polylinker sequence downstream of the core promoter region, which does not affect core promoter activity and allows the analysis of transcripts originating from TATA and TATA/INR variants in parallel by primer extension using the same radioactive primer. Transcription reactions (20 μl) were carried out at 30 °C for 1 h with the NE protein concentrations indicated. Transcripts from TATA and TATA/INR promoter variants were analyzed in parallel by primer extension using the same radiolabeled primer. Primer extension products were resolved by 6% denaturing PAGE and quantified by PhosphorImager analysis. C, immunoblot analysis of untreated NE (HeLa NE) and NE depleted of NC2 (NE[ΔNC2]). RNAP II was probed with antibody N-20, which recognizes an epitope at the N terminus of the largest subunit RPB1 and which reacts equally with CTD hyper-(IIO) and hypophosphorylated (IIA) RPB1 forms.
INR Function Counteracts NC2 Repression

A

\[
\begin{array}{c|cc|cc|cc|cc|cc|cc|cc|}
\text{mTdT} & \text{0.2 mg/ml NE} & \text{0.6 mg/ml NE} \\
\hline
\text{NE(\text{NC2})} & \text{1} & \text{2} & \text{5} & \text{1} & \text{2} & \text{5} \\
\text{NE(\text{NC2})} & 1.0 & 1.0 & 1.0 & 1.0 & 1.0 & 1.0 \\
\text{TATA/INR} & 0.5 & 0.5 & 0.5 & 0.5 & 0.5 & 0.5 \\
\text{TATA} & 0.5 & 0.5 & 0.5 & 0.5 & 0.5 & 0.5 \\
\end{array}
\]

B

\[
\begin{array}{c|cc|cc|cc|cc|cc|cc|}
\text{hsHSP70} & \text{1.8 mg/ml NE} & \text{8 mg/ml NE} \\
\hline
\text{NE(\text{NC2})} & \text{1} & \text{2} & \text{5} & \text{1} & \text{2} & \text{5} \\
\text{NE(\text{NC2})} & 1.0 & 1.0 & 1.0 & 1.0 & 1.0 & 1.0 \\
\text{TATA/INR} & 0.5 & 0.5 & 0.5 & 0.5 & 0.5 & 0.5 \\
\text{TATA} & 0.5 & 0.5 & 0.5 & 0.5 & 0.5 & 0.5 \\
\end{array}
\]

FIGURE 2. The INR element conferres resistance to repression by NC2. Transcription from A, mTdT and \(\&\), hsHSP70 promoter variants lacking or containing an INR element were analyzed in parallel as described in the legend to Fig. 1. Transcription reactions contained untreated HeLa NE or NC2-depleted HeLa nuclear extract (NE\(\&\)NC2) either at low protein concentration at which the INR element is inactive (A, 0.2 mg/ml) or at high concentrations at which INR function can be observed (A, 0.6 mg/ml; \(\&\), 1.8 mg/ml).

for 60 min at 30 °C in 40 \(\mu\)l of transcription buffer (35) containing 0.05% IGEPAL CA-630, 100 ng pGEM7Zf vector as a competitor DNA, and 0.1 mg/ml human insulin or bovine serum albumin. PICs were separated from unbound material on a magnetic separator and were washed once with transcription buffer containing 0.05% IGEPAL CA-630, 100 ng pGEM7Zf vector as a competitor DNA, and 0.1 mg/ml human insulin or bovine serum albumin. PICs were separated from unbound material on a magnetic separator and were washed once with transcription buffer containing 0.05% IGEPAL CA-630. One-half of the purified PICs was analyzed by in vitro transcription, the other half by immunoblotting.

RESULTS

Positive- and Negative-acting Factors Contribute to INR-selective Transcription in HeLa Nuclear Extracts—To investigate INR-mediated stimulation of basal transcription at TATA-containing promoters we compared the activity of simple model core promoters containing only a consensus TATA box (TATA) with variants containing both a consensus TATA box and an INR element (TATA/INR, Fig. 1). The well characterized murine terminal deoxynucleotidyltransferase promoter (mTdT) is a natural TATA-less promoter with a prototypical INR element as the only essential core promoter element (18). A mTdT-TATA/INR variant was obtained by introducing a consensus TATA box between positions −31 and −25 using site-directed mutagenesis (22). A mTdT-TATA core promoter variant with a TATA box as the only essential core promoter element was generated by introducing six nucleotide substitutions in the INR of mTdT-TATA/INR. These point mutations were previously shown to eliminate INR function without affecting transcription start site selection (22).

The human heat shock protein 70 gene core promoter (hsHSP70-TATA) contains a consensus TATA element 30 bp upstream of the transcription start and lacks an INR element (37). A hsHSP70 variant containing both TATA box and INR elements (hsHSP70-TATA/INR) was constructed by introducing the mTdT INR element at the hsHSP70 start site region using site-directed mutagenesis.

We used two-template in vitro transcription assays to determine the relative activity of corresponding TATA and TATA/INR promoter variants. Equimolar amounts of TATA-only and TATA/INR core promoter variants were transcribed in parallel and RNA products were analyzed quantitatively by primer extension analysis with the same radiolabeled primer (Fig. 1).

At a 10-fold lower nuclear extract concentration (0.2 mg/ml) compared with standard conditions mTdT-TATA and mTdT-TATA/INR variants are transcribed with similar activity (Fig. 2A, lane 1). Titrating increasing amounts of NE protein leads to dramatic stimulation of transcription from the mTdT-TATA/INR template but enhances transcription from the mTdT-TATA promoter only very moderately (Fig. 1A, lanes 2–4). At an NE protein concentration of 1.8 mg/ml, the mTdT-TATA/INR promoter is transcribed with ~100-fold higher activity than the mTdT-TATA variant lacking an INR element (Fig. 1A, lane 3). Similarly, strongly enhanced transcription activity of the hsHSP70-TATA/INR promoter relative to the corresponding hsHSP70-TATA variant could only be observed at NE protein concentrations of 1 mg/ml and above (Fig. 1B, lanes 3 and 4). Furthermore, at an intermediate NE protein concentration of 0.6 mg/ml (Fig. 1B, lane 2) the hsHSP70-TATA promoter is transcribed with 5-fold higher activity than its INR-containing counterpart HSP70-TATA/INR.

We point out that we observed similar results when we assayed the activity of TATA and TATA/INR promoter variants at varying NE protein concentrations site-by-site in separate reactions (data not shown). Thus the differences in the relative transcription activity of TATA/INR and TATA-only core promoters in response to increasing NE protein concentrations do not simply result from competition between these two promoters for a common limiting factor in our two-template transcription assays.

We infer from the results of the NE titration experiments shown in Fig. 1 the following. First, low amounts of NE protein fail to support stimulation of basal promoter activity by the INR. This situation is reminiscent of in vitro systems reconstituted with purified GTFs and RNAP II, which do not support INR function (23 and Fig. 4C). An INR-specific cofactor activity appears to be limiting in our HeLa nuclear extracts. It seems likely that this activity corresponds to a TAF- and INR-specific cofactor (TIC) described previously (23); however, this remains to be established.
Second, transcription from TATA-only promoters appears to be selectively repressed at higher NE protein concentrations. This is particularly evident from data obtained with hsHSP70 promoter variants (Fig. 1B), but can also be inferred from the fact that mTdT-TATA transcription is not significantly stimulated with increasing NE (Fig. 1A). A selective repression of transcription from promoter templates containing only a TATA box with increasing NE concentration was also noted in a previous study, in which the activity of TATA- and TATA/INR-containing promoter templates was compared in NE titration experiments (20).

Taken together, the results of titration experiments suggest that INR-enhanced basal transcription activity from TATA-containing promoters in human NE results from (i) a selective stimulation of basal transcription in the presence of the INR element and INR-specific cofactor activity (i.e. TIC) and, at the same time, (ii) a selective repression of transcription from TATA-containing promoters lacking an INR.

**INR-stimulated TATA-dependent Basal Transcription Is Resistant to NC2 Repression**—It was reported earlier that the NC2 (Drl1/DRAP1) complex, a ubiquitous repressor of TATA-dependent transcription (28, 31), inhibits basal transcription from diverse TATA-containing core promoters with varying efficiency (38). However, specific core promoter elements affecting differential repression by NC2 had not been identified. To test if NC2 repression is affected by the presence of an INR, we depleted NC2 from HeLa NE using a polyclonal antibody raised against the NC2α subunit. Depletion of NC2α was quantitative and resulted in co-depletion of ~90% of the NC2β subunit (Fig. 1C and data not shown). We found previously that an antibody for the NC2β subunit selectively depleted the CTD-phosphorylated RNAP IIo form along with NC2 (34). In contrast, RNAP II levels were not detectably affected when HeLa NE was depleted with our antibody raised against the NC2α subunit (Fig. 1C). It is possible that our NC2α antibody disrupts interactions between the RNAP IIo form and NC2.

Comparative analysis of untreated NE and NC2-depleted NE (NE[ΔNC2]) in titration experiments revealed that at low NE protein concentrations, which do not support INR function, NC2 depletion caused a similar de-repression of TATA-only and TATA/INR promoter variants (Fig. 1, compare lanes 1 and 5; Fig. 2A, compare lanes 1 and 2). In contrast, at higher NE protein concentrations supporting INR stimulation of basal transcription levels, depletion of NC2 selectively increased transcription from TATA-only promoter templates, but had comparatively little effect on the activity of the corresponding TATA/INR variants (Fig. 1, compare lanes 2–4 with lanes 6–8; Fig. 2A, lanes 6 and 7; Fig. 2B, lanes 1 and 2). A selective de-repression of the TATA-only variant upon NC2 depletion was particularly evident at the minimal NE protein concentration at which INR function could be observed: in the presence of 0.6 mg/ml NE protein the mTdT-TATA/INR promoter has ~8–10fold higher activity than the mTdT-TATA promoter variant (Figs. 1A, lane 2 and 2A, lane 6). Under these conditions NC2 depletion increases mTdT-TATA promoter activity 12–16-fold, but increases mTdT-TATA/INR promoter activity only 1.5–2-fold (compare Figs. 1A, lanes 2 and 6 and 2A, lanes 6 and 7). As a result, INR-selective promoter activity is lost and mTdT-TATA and mTdT-TATA/INR promoters are transcribed with similar activity (Fig. 1A, lane 6). Similarly, hsHSP70-TATA promoter activity is stimulated 3fold in the presence of an INR at a NE protein concentration of 1.2 mg/ml (Fig. 1B, lane 3). Upon NC2 depletion transcription from hsHSP70-TATA increases more than 8-fold, but hsHSP70-TATA/INR activity increases only 2-fold and, as a result, HSP70-TATA and hsHSP70-TATA/INR variants are transcribed with similar activity (Fig. 1B, compare lanes 3 and 7). These results suggested that the presence of an INR prevents NC2 repression, provided positive-acting INR-specific co-factor (TIC) activity is available.

To confirm these observations, we performed add-back experiments and supplemented NE[ΔNC2] with highly purified recombinant human NC2 complex (rNC2). At a limiting NE protein concentration (0.2 mg/ml), which does not support INR function (Fig. 2A, lanes 1 and 2), addition of rNC2 to NE[ΔNC2] repressed transcription from both mTdT-TATA and mTdT-TATA/INR core promoter variants with similar efficiency (Fig. 2A, lanes 2–5). In contrast, at a NE extract concentration that supports INR-stimulated transcription levels (0.6 mg/ml; Fig. 2A, lane 6) added rNC2 selectively repressed transcription from the mTdT-TATA promoter lacking an INR, but did not significantly affect transcription from the mTdT-TATA/INR core promoter variant (Fig. 2A, lanes 7 and 10). Similarly, added rNC2 selectively repressed transcription from the hsHSP70 TATA-only promoter at NE protein concentration supporting INR-selective transcription of the hsHSP70-TATA/INR variant (1.8 mg/ml; Fig. 2B). These data clearly demonstrate that INR-stimulated basal transcription from TATA-containing promoters is resistant to NC2 repression.

**NC2 Does Not Repress TATA-less Transcription Directed by the INR Element**—The specific TATA box binding activity of TBP appears not to be required for INR-directed transcription from the TATA-less mTdT promoter (25), suggesting that transcription initiation at the mTdT promoter does not involve the formation of a cognate TBP/DNA nucleoprotein complex as seen on TATA-containing promoters. On the other hand, human NC2 was shown to interact with TBP bound to TATA-less DNA sequences in a recent study (39). In addition, *Drosophila* NC2 was previously identified as a positive cofactor for transcription of TATA-less promoters containing a DPE (40). To test if INR-mediated TATA-less transcription in NE is affected by NC2 we compared basal transcription levels from the natural TATA-less mTdT core promoter (mTdT-INR) in untreated NE and in NE[ΔNC2]. We find that transcription from the TATA-less mTdT core promoter is unaffected by NC2 depletion (Fig. 3). Thus, human NC2 appears not to affect INR-mediated transcription, at least under our experimental conditions.

**INR-mediated Resistance to NC2 Repression Requires TFIID TAFs**—Previous studies had established that TFIID TAF subunits are essential for INR activity (21, 22, 24, 25). To test if TAFs are also required for INR-mediated resistance to NC2 repression, we prepared HeLa NE lacking TFIID (NE[ΔD]) and HeLa NE lacking both TFIID and NC2 (NE[ΔDΔNC2]). HeLa NE and NE[ΔNC2] were passed over anti-TBP and anti-TAF5 antibody columns to remove TBP and TFIID TAFs as described.
Previously (35). Transcription in NE[ΔD] was completely dependent on highly purified recombinant 6His:tagged TBP (6His:TBP) or immunoaffinity-purified FLAG:tagged human TFIID complex (f:TFIID; Fig. 4, A and B). 6His:TBP supported similar levels of basal transcription from TATA-only and TATA/INR mTdT promoter variants (Fig. 4A, lane 2). In contrast, addition of equivalent TBP amounts of f:TFIID resulted in strong preferential transcription of the TATA/INR promoter, whereas transcription from the TATA-only promoter variant was barely detectable (Fig. 4A, lane 3).

These data confirm that TFIID TAFs are essential for INR-selective transcription, consistent with results of earlier studies using NE in which TFIID was selectively inactivated by heat-treatment (21, 22, 24, 25). The data further corroborate results of earlier studies suggesting that TFIID TAFs negatively affect the ability of TBP to drive TATA-dependent transcription initiation in a core promoter-dependent manner (35, 41).

Next we examined the ability of purified rNC2 complex to repress transcription from TATA- and TATA/INR-containing mTdT promoter variants in NE[ΔD]NC2 in the presence of 6His:TBP or f:TFIID (Fig. 4B). Under the conditions added 6His:TBP supported about 2-fold higher transcription levels from the TATA-only promoter compared with the TATA/INR core promoter variant (Fig. 4B, lane 2), whereas added f:TFIID supported almost 2-fold higher levels of transcription from the TATA/INR promoter compared with its TATA-only counterpart (Fig. 4B, lane 5). Added rNC2 repressed transcription from TATA-only and TATA/INR promoter variants with similar efficiency when NE[ΔD]NC2 was supplemented with 6His:TBP (Fig. 4B, lanes 2–4). In contrast, rNC2 selectively repressed TATA-only transcription without significantly affecting TATA/INR transcription in the presence of f:TFIID (Fig. 4B, lanes 5–7). These data clearly demonstrate that TFIID TAFs are essential for INR-mediated resistance to NC2 repression.

Finally INR function is not observed when transcription is reconstituted with purified GTFs and RNAP II (Fig. 4C, lanes 1 and 5). In the purified system NC2 represses transcription from TATA and TATA/INR promoter templates with similar efficiency when TFIID TAFs are present (Fig. 4C). Thus, TFIID TAFs appear not to be sufficient to confer resistance to transcription repression by NC2 at TATA/INR promoters when INR-specific cofactor activity is lacking and the INR is not functional.

FIGURE 3. INR-dependent TATA-less transcription is unaffected by NC2.

INR Function Counteracts NC2 Repression

AUGUST 24, 2007

24772

VOLUME 282 • NUMBER 34 • JOURNAL OF BIOLOGICAL CHEMISTRY

TAF-dependent INR Element Activity Reduces NC2 Promoter Occupancy—How does TAF- and TIC-dependent INR activity counteract NC2 repression? To gain further insight into this question we performed immobilized template recruitment assays. Linear 5′-biotinylated DNA templates containing mTdT-TATA or mTdT-TATA/INR core promoter variants were produced by PCR, gel-purified and attached to streptavidin-coated magnetic beads. The immobilized promoter templates were incubated with TFIID-depleted HeLa nuclear extract (NE[ΔD]) supplemented with either recombinant human 6His:TBP or immunoaffinity-purified human f:TFIID complex to assemble PICs. PICs were separated from unbound NE proteins, washed, and protein composition and transcription activity of isolated PICs was analyzed in parallel by immunoblotting and by single round transcription (Fig. 5A).

Consistent with earlier template recruitment studies by others (42), only a small fraction of isolated PICs were transcriptionally active. Nevertheless, we observed overall a very good correlation between the amount of PIC components detected on immobilized templates and transcription activity (Figs. 5B and 6B). In the presence of 6His:TBP PICs isolated on TATA-only and TATA/INR templates contained comparable amounts of GTFs, RNAP II, and NC2 and supported similar levels of transcription (Fig. 5B, lanes 3 and 4). In contrast, when NE[ΔD] was supplemented with purified f:TFIID complex PICs assembled on the mTdT-TATA/INR promoter supported 4-fold higher transcription levels compared with PICs assembled on the mTdT-TATA promoter (Fig. 5B, lanes 5 and 6). The increased transcription activity of PICs assembled in the presence of INR and TAFs correlated with enhanced recruitment of GTFs and RNAP II (Fig. 5B, lanes 5 and 6). Most strikingly, strongly enhanced recruitment of TFIIA and TFIIIB in the presence of an INR and TAFs was accompanied by a corresponding loss of NC2 binding (Fig. 5B, lanes 5 and 6). Biochemical studies (26, 28, 29, 31, 33) and studies in yeast (43) have shown that NC2 and TFIIA compete for binding to TBP/TATA complexes, and that NC2 binding blocks recruitment of TFIIB. Our data suggest that INR activity changes the equilibrium between TFIIA, TFIIIB, and NC2 in a TAF-dependent manner resulting...
in preferential binding of TFIIA and TFIIIB over competing NC2 to promoter-bound TBP.

The enhanced recruitment of GTFs and RNAP II, in particular of TFIIA and TFIIIB, to promoter-bound TFIID observed in response to TAF-dependent INR activity is reminiscent of activator-stimulated recruitment of GTFs to immobilized templates (44–46). To investigate if stimulation of PIC assembly by promoter-specific activators in vitro also coincides with changes in NC2 promoter binding, we performed recruitment assays with immobilized HIV-1 promoter templates containing (i) the HIV-1 core promoter from –33 to +80 and (ii) the HIV-1 core promoter in the context of its natural HIV-1 enhancer sequences up to position –111 (Fig. 6A), which contain binding sites for Sp1 and NFκB and which are sufficient to strongly activate HIV-1 transcription in vivo (34). As expected, the presence of the HIV-1 enhancer strongly enhanced PIC assembly and transcription at immobilized HIV-1 promoter templates. Remarkably, activator-induced PIC assembly correlated with strongly enhanced recruitment of TFIIA and with a corresponding loss of NC2 binding (Fig. 6B), very similar to INR-stimulated PIC assembly. Thus TAF-dependent INR activity and at least some transcription activators appear to stimulate functional PIC assembly in a similar fashion.

DISCUSSION

We investigated the molecular mechanisms underlying stimulation of basal transcription from TATA-containing promoters by the INR element. INR function is not supported by in vitro systems reconstituted with purified RNAP II and GTFs. So-called TAF- and INR-specific cofactor (TIC) activity is required (23), which has not yet been biochemically defined. Because a defined system is not available, investigations into the mechanisms underlying INR-directed basal transcription have to be conducted in crude systems containing the required cofactor(s).

In HeLa NEs, which contain a close-to physiological complement of human nuclear factors, the presence of an INR strongly enhances basal transcription from TATA-dependent promoters, provided TATA and INR are within a distance that permits TATA-INR cooperativity (19). The key finding in this study is that TATA-directed basal transcription becomes resistant to the ubiquitous transcription repressor NC2 (Dr1/DRAP1) when stimulated by an INR. Thus the strongly enhanced basal activity of TATA-containing promoters in the presence of an INR is caused by simultaneous action of positive- and negative-acting cofactors: (i) a TAF- and INR-dependent cofactor activity, which selectively stimulates transcription in the presence of the INR, and (ii) NC2, which selectively represses transcription from TATA-containing promoters lacking an INR.

Repression of TATA-mediated RNAP II transcription initiation by NC2 has been extensively characterized. NC2 directly interacts with the TBP/TATA box nucleoprotein complex and prevents recruitment of TFIIA and TFIIIB (26, 28, 29, 31, 32). Biochemical studies have shown that NC2 binding to the TBP/TATA complex is competitive with TFIIA (28, 31, 33). Consistent with in vitro observations, results of genetic studies suggest that NC2 and TFIIA are in dynamic equilibrium in budding yeast (43). In this study, we find that TAF-dependent INR activity strongly increases recruitment of TFIIA and TFIIIB to TATA-containing promoters and, at the same time, interferes with NC2 binding during PIC assembly. Thus TAF-dependent INR activity appears to change the dynamic equilibrium of
TFIIA and NC2 binding to TBP during PIC assembly in favor of TFIIA. We find in template recruitment assays that recombinant NC2 complex (rNC2) binds with similar affinity to human f:TTFIID bound to TATA-only or TATA/INR core promoter variants (data not shown). Thus, the presence of an INR sequence appears not to affect binding of rNC2 to TFIID/promoter complexes in the absence of other factors. Furthermore, rNC2 represses TATA-only and TATA/INR model promoters with similar efficiency when transcription is reconstituted with f:TTFIID, highly purified GTFs and RNAP II. These results suggest that INR-mediated resistance to NC2 repression requires both TAFs and INR-specific cofactor activity.

NC2 was originally identified as a repressor activity in human nuclear extract fractions that, at low concentrations, selectively inhibited basal promoter activity but not Sp1-activated transcription, thereby causing an increased amplitude of Sp1 induction (26). Subsequent studies in yeast and in mammalian cells have provided further evidence that transcription induction by some activators involves reversal of NC2 repression (47–52). Consistent with these studies we show that stimulation of HIV-1 core promoter activity by cognate HIV-1 enhancer sequences correlates with enhanced recruitment of RNAP II basal machinery components, in particular TFIIA, and with a corresponding loss of NC2 binding during PIC assembly.

Gene activation in metazoan cells is typically achieved through concerted action of multiple activators, which at least at some enhancers can form higher order nucleoprotein structures termed enhanceosomes (53, 54). Different activators can function cooperatively during gene induction by targeting distinct steps of the transcription process, including the recruitment of GTFs and RNAP II, the assembly of a functional PIC, and steps during the initiation and elongation phases (55). The intrinsic ability of the
basal RNAP II machinery to initiate transcription is determined by the quality of the core promoter sequence and varies greatly between promoters. Hence, one obvious model to explain differences in the amplitude by which different genes respond to various activators and repressors (1, 2, 9) is that rate-limiting steps in the assembly and function of the basal RNAP II transcription apparatus that can be affected by various activators/repressors differ depending on the core promoter architecture.

The activity of the basal RNAP II machinery is further modulated by a complex array of positive- and negative acting (co)factors, which act in conjunction with sequence-specific activators and repressors and which are essential for stringent regulation of gene activity and optimal levels of induction in response to environmental cues (27, 56). In this work we show that a functional INR core promoter counteracts repression of TATA-containing promoters by the well-characterized negative cofactor NC2. Thus core promoter sequence can also affect the ability of negative-acting (and presumably also positive-acting) cofactors to modulate the intrinsic activity of the basal RNAP II apparatus.

We note remarkable similarities between the stimulation of TATA-dependent promoter activity by the INR core promoter element and by enhancer-bound transcription activators; in both cases enhanced assembly of functional PICs correlated with increased recruitment of TFIIA and TFIIB and a corresponding loss of NC2 binding. The precise molecular mechanisms underlying INR-directed transcription initiation are still unknown. However, it has been clearly shown that the functional readout of the INR sequence requires TAF subunits within TFIID (21, 22, 24, 25). Our data demonstrate that TAFs are also required for INR-mediated resistance to NC2 repression. TAFs within human TFIID have been shown to interact with promoter DNA downstream of the TATA box region up to about position +35 relative to the transcription start site (21, 57–60). TFIID downstream promoter interactions do not require an INR sequence in the context of complex core promoters such as the Ad2ML promoter (59). However, specific TAF-INR interactions have been demonstrated at simple TATA-only model promoters, where the presence of an INR sequence induces interactions of human TFIID with promoter DNA at and around the transcription start site (21, 61) and increases TFIIID binding in Mg2+ agarose EMSA assays in a TFIIA-dependent manner (61). Importantly, activators have also been shown to induce TFIID interactions with promoter regions downstream of the TATA box region. It was shown that activator-induced isomerisation of the TFIID/promoter complex requires TFIIA and results in an increased ability of TFIIID to recruit TFIIIB and to assemble an active RNAP II transcription initiation complex (62). We speculate that, similar to activators, TAF-INR interactions in conjunction with INR-specific coactivator activity may induce and/or stabilize a specific conformation of the TFIID/promoter complex that favors recruitment of TFIIA and TFIIIB over competing NC2. Identification of the INR-specific coactivator activity responsible for INR-mediated stimulation of TATA-dependent transcription will be crucial to further investigate this interesting possibility.

Acknowledgments—We thank Michael Meisterernst for NC2α and NC2β bacterial expression vectors, Robert G. Roeder for hTFIIA, -B, and -IE bacterial expression vectors, for antibodies to hsTFB, TAFS, TFIIIE, TFIIA/α, TFIIH p62, and MED21 (Srb7) and for the HeLa 3–10 cell line expressing fTFB, Yoshitaki Okuma for the hTFIIIF bacterial expression vector, Alexander Hoffmann for human TAF12 antibody, and Jörg Kaufmann for human TAF2 antibody.

REFERENCES
1. Gross, P., and Oelgeschläger, T. (2006) Biochem. Soc. Symp. 73, 225–236
2. Smale, S. T., and Kadonaga, J. T. (2003) Annu. Rev. Biochem. 72, 449–479
3. Ohler, U., Liao, G. C., Niemann, H., and Rubin, G. M. (2002) Genome Biol. 3, RESEARCH0087
4. Suzuki, Y., Tsunoda, T., Sese, J., Taira, H., Mizushima-Sugano, J., Hata, H., Ota, T., Isogai, T., Tanaka, T., Nakamura, Y., Sayama, A., Sakaki, Y., Morishita, S., Okubo, K., and Sugano, S. (2001) Genome Res. 11, 677–684
5. Bajic, V. B., Choudhary, V., and Hock, C. K. (2004) In Silico Biol. 4, 109–125
6. Gershenson, N. I., and Ishikhes, I. P. (2005) Bioinformatics 21, 1295–1300
7. Butler, J. E. F., and Kadonaga, J. T. (2001) Genes Dev. 15, 2515–2519
8. Butler, J. E. F., and Kadonaga, J. T. (2002) Genes Dev. 16, 2583–2592
9. Smale, S. T. (2001) Genes Dev. 15, 2503–2508
10. Ohtsuki, S., Levine, M., and Cai, H. N. (1998) Genes Dev. 12, 547–556
11. Bushnell, D. A., Westover, K. D., Davis, R. E., and Kornberg, R. D. (2004) Science 303, 983–988
12. Hahn, S. (2004) Nat. Struct. Mol. Biol. 11, 394–403
13. Thomas, M. C., and Chiang, C. M. (2006) Crit. Rev. Biochem. Mol. Biol. 41, 105–178
14. Roeder, R. G. (1996) Trends Biochem. Sci. 21, 327–335
15. LaGrange, T., Kapanidis, A. N., Tang, H., Reinberg, D., and Ehrlich, R. H. (1998) Genes Dev. 12, 34–44
16. Evans, R., Fairley, J. A., and Roberts, S. G. E. (2001) Genes Dev. 15, 2945–2949
17. Deng, W., and Roberts, S. G. (2005) Genes Dev. 19, 2418–2423
18. Smale, S. T., and Baltimore, D. (1989) Cell 57, 103–113
19. O’Shea-Greenfield, A., and Smale, S. T. (1992) J. Biol. Chem. 267, 1391–1402
20. Zenzie-Gregory, B., O’Shea-Greenfield, A., and Smale, S. T. (1992) J. Biol. Chem. 267, 2823–2830
21. Kaufmann, J., and Smale, S. T. (1994) Genes Dev. 8, 821–829
22. Martinez, E., Chiang, C.-M., Ge, H., and Roeder, R. G. (1994) EMBO J. 13, 3115–3126
23. Martinez, E., Ge, H., Tao, Y., Yuan, C.-X., Palhan, V., and Roeder, R. G. (1998) Mol. Cell Biol. 18, 6571–6583
24. Smale, S. T., Schmidt, M. C., Berk, A. J., and Baltimore, D. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4509–4513
25. Martinez, E., Zhou, Q., L’Etoile, N. D., Oelgeschläger, T., Berk, A. J., and Roeder, R. G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11864–11868
26. Meisterernst, M., and Roeder, R. G. (1991) Cell 67, 557–567
27. Roeder, R. G. (1998) Cold Spring Harb. Symp. Quant. Biol. 63, 201–218
28. Mermelstein, F., Yeung, K., Cao, J., Inostroza, J. A., Eidelberg-Bromage, H., Eagelson, K., Landsman, D., Levitt, P., Tempst, P., and Reinberg, D. (1996) Genes Dev. 10, 1033–1048
29. Inostroza, J. A., Mermelstein, F. H., Ha, I., Lane, W. S., and Reinberg, D. (1992) Cell 70, 477–489
30. Goppelt, A., and Meisterernst, M. (1996) Nucleic Acids Res. 24, 4450–4455
31. Goppelt, A., Stelzer, G., Lottspeich, F., and Meisterernst, M. (1996) EMBO J. 15, 3105–3116
32. Kamada, K., Shu, F., Chen, H., Malik, S., Stelzer, G., Roeder, R. G., Meisterernst, M., and Burley, S. K. (2001) Cell 106, 71–81
33. Kim, T. K., Zhao, Y., Ge, H., Bernstein, R., and Roeder, R. G. (1995) J. Biol. Chem. 270, 10976–10981
34. Castaño, E., Gross, P., Wang, Z., Roeder, R. G., and Oelgeschläger, T.
INR Function Counteracts NC2 Repression

(2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7184–7189
35. Oelgeschläger, T., Tao, Y., Kang, Y. K., and Roeder, R. G. (1998) Mol. Cell 1, 925–931
36. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475–1489
37. Drabent, B., Genthe, A., and Benecke, B. J. (1986) Nucleic Acids Res. 14, 8933–8948
38. Kim, J., Parvin, J. D., Shykind, B. M., and Sharp, P. A. (1996) J. Biol. Chem. 271, 18405–18412
39. Gilfillan, S., Stelzer, G., Piaia, E., Hofmann, M. G., and Meisterernst, M. (2004) J. Biol. Chem. 280, 6222–6230
40. Willy, P. I., Kobayashi, R., and Kadonaga, J. T. (2000) Science 290, 982–985
41. Guermah, M., Tao, Y., and Roeder, R. G. (2001) Mol. Cell Biol. 21, 6882–6894
42. Yudkovsky, N., Ranish, J. A., and Hahn, S. (2000) Nature 408, 225–229
43. Xie, J., Collart, M., Lemaire, M., Stelzer, G., and Meisterernst, M. (2000) EMBO J. 19, 672–682
44. Johnson, K. M., Wang, J., Smallwood, A., Arayata, C., and Carey, M. (2002) Genes Dev. 16, 1852–1863
45. Kim, T. K., Kim, T. H., and Maniatis, T. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12191–12196
46. Lin, Y. S., and Green, M. R. (1991) Cell 64, 971–981
47. Prelich, G. (1997) Mol. Cell Biol. 17, 2057–2065
48. Gadbois, E. L., Chao, D. M., Reese, J. C., Green, M. R., and Young, R. A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3145–3150
49. Kraus, V. B., Inostroza, J. A., Yeung, K., Reinberg, D., and Nevins, J. R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6279–6282
50. Kim, S., Na, J. G., Hampsey, M., and Reinberg, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 820–825
51. Caswell, R., Bryant, L., and Sinclair, J. (1996) J. Virol. 70, 4028–4037
52. Yeung, K. C., Inostroza, J. A., Mermelstein, F. H., Kannabiran, C., and Reinberg, D. (1994) Genes Dev. 8, 2097–2109
53. Carey, M. (1998) Cell 92, 5–8
54. Merika, M., and Thanos, D. (2001) Curr. Opin. Genet. Dev. 11, 205–208
55. Lemon, B., and Tijan, R. (2000) Genes Dev. 14, 2551–2569
56. Roeder, R. G. (2005) FEBS Lett. 579, 909–915
57. Nakajima, N., Horikoshi, M., and Roeder, R. G. (1988) Mol. Cell Biol. 8, 4028–4040
58. Zhou, Q., Boyer, T. G., and Berk, A. J. (1993) Genes Dev. 7, 180–187
59. Chiang, C.-M., Ge, H., Wang, Z., Hoffmann, A., and Roeder, R. G. (1993) EMBO J. 12, 2749–2762
60. Oelgeschläger, T., Chiang, C.-M., and Roeder, R. G. (1996) Nature 382, 735–738
61. Emami, K. H., Jain, A., and Smale, S. T. (1997) Genes Dev. 11, 3007–3019
62. Chi, T., and Carey, M. (1996) Genes Dev. 10, 2540–2550