Promoter Influences Transcription Elongation

TATA-BOX ELEMENT MEDIATES THE ASSEMBLY OF PROCESSIVE TRANSCRIPTION COMPLEXES RESPONSIVE TO CYCLIN-DEPENDENT KINASE 9

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Pausing of RNA polymerase II (RNAPII) during transcript elongation is an important mechanism for regulating gene expression at many genes. In this study, we investigated the mechanism of regulated elongation of c-myc and human immunodeficiency virus-1 (HIV-1) using an in vitro elongation assay that reproduces the conditional block to elongation. We found that HIV-1 Tat can activate the RNAPII transcription complexes paused on c-myc by enhancing their elongation efficiency. We determined that cyclin-dependent kinase 9 (CDK9), the kinase subunit of positive transcription elongation factor b (P-TEFb) complex, regulates transcriptional elongation of c-myc and is present in transcription pre-initiation complexes formed on the c-myc promoter, which emphasizes a common mechanism of elongation control between HIV-1 and c-myc genes.

We also investigated the roles of upstream elements of the HIV-1 and c-myc promoters in CDK9-activated transcriptional elongation. We found that the TATA-box element mediates the assembly of processive transcription complexes responsive to CDK9 and that specific combinations of upstream activation binding sites contribute to the recruitment of these complexes. We propose a common mechanism for elongation control of the c-myc and HIV-1 genes with an essential role for the TATA-box and specific modulatory contribution of upstream regulatory sequences, derived from the unique structure of the promoters, to form a composite surface for efficient recruitment of elongation-competent transcription complexes.

The once commonly held view that promoters regulate transcription only by recruiting RNAPII initiation complexes has been challenged by recent findings that demonstrate a significant cross-talk between events occurring at the promoter and the process of elongation. For example, activation domains of factors that can promote the formation of pre-initiation complexes (PIC) can affect elongation and mRNA splicing events (1–3). Also, the Brm component of the SWI/SNF complex involved in chromatin remodeling on promoters dramatically affects alternative splicing choice decisions by modifying RNAPII elongation rate (4). Therefore, promoter structure and occupancy by transcription factors can affect the process of elongation and coupled mRNA processing events.

Elongation can be regulated by both positive and negative acting factors. For instance, negative transcription elongation factors such as the 5,6-dichloro-1β-D-ribofuranosylbenzimidazole-sensitive inducing factor and the negative elongation factor (5, 6) can enhance pausing and/or arrest of RNAPII elongation complexes. Positive transcription elongation factors such as positive transcription elongation factor b (P-TEFb) enhance the overall rate of transcription elongation. P-TEFb is composed of the CDK9 kinase and cyclin T1 (7). At present, the mode of action of these negative and positive-acting factors is not well understood. Negative transcription elongation factors somehow pause RNAPII complexes in the 5′ region of the transcription unit, resulting in arrested transcription, whereas positive transcription elongation factors overcome the effects of negative transcription elongation factors by releasing RNAPII complexes from its arrest (5, 6). This is in part accomplished by multiple phosphorylation events of the carboxyl-terminal domain of the largest subunit of the RNAPII. A hyperphosphorylated carboxyl-terminal domain correlates with the presence of RNAPII at the coding regions of genes and, thus, is considered a mark of elongating polymerases (8).

Among the most notable examples of promoter-proximal pausing, where RNAPII complexes are stalled in the 5′ region of the transcription unit, are the human immunodeficiency virus type 1 (HIV-1) genes and the proto-oncogene c-myc. HIV-1 regulates its gene expression through the interplay of specific DNA cis-acting elements located within the long terminal repeat (LTR), the trans-acting viral protein Tat, and cellular factors. Tat activates viral gene expression by enhancing the elongation efficiency of RNAPII complexes in a process that is

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mediated by the interaction of Tat with P-TEFb, which enhances RNAPII elongation by phosphorylating the carboxy-terminal domain of RNAPII (9). Tat activates transcription through an unusual mechanism; it binds to an RNA element designated TAR (for trans-activation response element) in the 5’ end of the HIV-1 transcript.

The c-myc proto-oncogene encodes a DNA-binding protein that is involved in the control of cellular proliferation, and its expression is altered in a wide variety of human tumors including breast, colon, cervical carcinomas, small lung carcinomas, osteosarcomas, glioblastomas, and myeloid leukemias (10). In normal cells, c-myc RNA is expressed at high levels during proliferative growth, but its levels decrease significantly during cellular differentiation. The decrease in c-myc RNA levels has been attributed to mechanisms operating at the level of elongation. In vivo RNAPII footprinting experiments have shown promoter-proximal pausing of transcription complexes in the c-myc gene (11), which suggests that the previously described block to elongation is due to paused RNAPII complexes in the c-myc promoter region (12). In this context it is possible that the phosphorylation state of the RNAPII carboxy-terminal domain might contribute to the promoter proximal pausing of transcription complexes. The mechanism and factors that regulate transcriptional elongation and promoter-proximal pausing of c-myc, however, remain to be elucidated.

The pausing of RNAPII at discrete sites in the 5’ region of the transcription unit is a widespread phenomenon that takes place at many other genes (for review, see Ref. 13), and based upon similarities in elongation control among some of these genes, a model for control of expression of these genes at the elongation phase of transcription has been proposed (12, 14). This model suggests that distinct promoter elements can nucleate the assembly of RNAPII transcription complexes that differ in their elongation efficiency. However, the influence that promoters have on promoter-proximal pausing and elongation factor recruitment has not been investigated yet.

In the study presented here we have developed an in vitro transcription elongation assay that reproduces promoter-proximal pausing of c-myc gene. We used this assay to carry out an analysis of the regulation of the c-myc and HIV-1 transcription elongation. We found that HIV-1 Tat can activate the c-myc-paused RNAPII transcription complexes, which are then able to elongate more efficiently. We also found that CDK9 regulates transcription elongation of c-myc and show that this factor is present in pre-initiation complexes formed on the c-myc promoter. Finally, we analyzed the different roles of upstream elements of the HIV-1 and c-myc promoters in CDK9-activated elongation by targeted recruitment of this cyclin-dependent kinase in vivo. In our experiments changes engineered into the HIV-1 and c-myc TATA-box sequences greatly alter CDK9 transcriptional induction, which suggest an essential role of this element in nucleate active, elongation-competent transcription complexes. In addition, the specific combinations of upstream activator binding sites present at each promoter contribute to this process by modulating the efficiency of the recruitment of these complexes to the TATA-box. Our data argue that proteins bound to the TATA-box and upstream sequences make simultaneous and stereospecific contacts that are necessary for efficient recruitment of processive transcription complexes.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The parental template HIVdGless was constructed by inserting a PCR fragment containing HIV LTR sequences (from −133 to +120) into KpnI/XbaI-digested pTZHIVdGless plasmid (15). The HIVBdGless contains a deletion of the TAR bulge. In the HIV(TATA)mcdGless plasmid the TATA-box has been changed to GTCAC by standard techniques. The TdTGless plasmid contains the initiator element (−6 to +11) from the TATA-less promoter of the TdT gene. The parental P1P2cmycdGless and P2cmycdGless plasmids were constructed by inserting PCR fragments containing human c-myc sequences (from −346 to +42 and from −94 to +42, respectively) into KpnI/XbaI-digested pTZHIVdGless plasmid. Human c-myc DNA was kindly provided by Ana Aranda (Instituto de Investigaciones Biomédicas “Alberto Sols,” Madrid, Spain). The c-mycTARdGless plasmids were constructed by inserting PCR fragments containing the HIV TAR or mutated bulge sequences (from +1 to +120) into the parental plasmids by standard techniques. Oligonucleotide sequences and protocols are available upon request.

For the transient transfection experiments, the G5HIV-LUC reporter plasmid was constructed by inserting HIV sequences (from −133 to +120) into MluI/NheI-digested G5pGL3 vector. G5pGL3 vector was created by inserting five repeats of GAL4 DNA-binding sequences between KpnI and SacI sites of the G5pGL3 vector. The parental pGL3 vector was constructed by inserting HIV sequences from KpnI/XbaI-digested HIVdGless plasmid. Human c-myc DNA was kindly provided by Ana Aranda (Instituto de Investigaciones Biomédicas “Alberto Sols,” Madrid, Spain). The c-mycTARdGless plasmids were constructed by inserting PCR fragments containing the HIV TAR or mutated bulge sequences (from +1 to +120) into the parental plasmids by standard techniques. Oligonucleotide sequences and protocols are available upon request.

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**Antibodies and Recombinant Proteins**—Antibodies against CDK9, cyclin T1, RNAPII (N-20), TFIIID (TATA-box-binding protein (TBP)), CDK2, nucleolin, and rabbit IgGs were all purchased from Santa Cruz Biotechnology, Inc. Antibodies against U2AF65 were kindly provided by J. Valcárcel (CRG, Barcelona, Spain). The CA150 antibodies used in this study were described previously (21). Antibodies against Sp1 were originally pro-
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vided by J. Horowitz (Duke University Medical Center, Durham, NC). Anti-Tat antibodies were a generous gift of K. T. Jeang, and anti-GFP antibodies were from Invitrogen. Expression and purification of recombinant Tat and P-TEFb proteins have been described previously (15, 22).

**Purification of PICs**—25 μl of streptavidin-coated magnetic beads (Dynabeads; Dynal) pre-equilibrated in 25 μl of binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM NaCl) were incubated with 250 ng of biotinylated templates for 30 min at room temperature. DNA-containing beads were subsequently incubated in IVT buffer (14 mM HEPES, pH 7.9, 14% glycerol, 7 mM MgCl2, 1 mM dithiothreitol, 0.1 mM EDTA, 10 mM creatine phosphate, 250 ng of poly(I)-poly(C), 300 ng of poly(diI)-poly(diC), and phenylmethylsulfonyl fluoride at 100 μg/ml) containing 68 mM KCl (IVT-68) and 1% bovine serum albumin for 30 min at room temperature. After the blocking step beads were washed with IVT-68 buffer containing 0.05% Nonidet P-40 and incubated with 10 μl of HeLa nuclear extract (8 μg/μl) prepared by the method of Dignam et al. (23) in a reaction mixture (25 μl) containing IVT-68 buffer for 30 min at 30 °C. HeLa nuclear extract was first treated with 0.1 μg/ml TaqI-digested pG3 plasmid (Promega) to minimize nonspecific binding. The immobilized templates were then harvested using a magnetic device, and the PICs were washed extensively with IVT buffer containing 150 mM KCl (IVT-150) and 0.1% Nonidet P-40. In vitro transcription and Western blot analysis could then be performed using the purified PICs assembled on the immobilized templates.

**In Vitro Transcription/Elongation Assay**—The in vitro transcription/elongation assay was performed as previously described (15). The trans-activation of short and long transcripts was calculated by measuring the radioactivity for each product with phosphorimaging. Data were normalized for uridine content by dividing the radioactivity of the product by the number of uridine residues in the fragment (24 for the 83-nt transcript). The transfection, and pSV2GAL reporter vector CMV-CAT was used as an internal control for transfection, and pSV2GAL plasmid was used to keep constant the total amount of nucleic acid.

**RESULTS**

The study of the mechanisms governing transcriptional elongation has been hindered by the lack of appropriate methods to measure RNAPII efficiency. We, therefore, sought to establish an in vitro system that would allow us to biochemically and functionally dissect the regulation of transcription elongation due to promoter proximal pausing in the human c-myc gene. Transcription driven by the c-myc promoter can be observed in vitro by using HeLa nuclear extracts (26). In those in vitro cell-free assays, transcription from the c-myc promoter is measured as run-off products. To directly measure the elongation efficiencies of transcribing polymerases, we established an in vitro elongation assay that utilizes a double G-less cassette template (24) driven by the c-myc promoter. This template synthesizes transcripts that contain two regions (cassettes) of different sizes that lack guanosine residues; therefore, these G-less cassettes are resistant to digestion with RNase T1. One G-less cassette is located proximal to the promoter so as to measure the numbers of transcription complexes that reach nt +146 (short), and the second, downstream, G-less cassette measures the number of transcripts beyond nucleotide +1910 (long) (see
cells (27). Thus, we decided to construct two different c-myc promoter templates containing either the P2 promoter alone or both the P1 and P2 promoters (Fig. 1A).

Transcription from the c-myc promoters was efficient (Fig. 1B, lanes 1 and 4), reflecting efficiency of RNAPII complexes formed on the c-myc promoter. However, increasing the concentration of DNA template in the transcription reaction dramatically reduced the levels of c-myc elongation as observed by the increase in the amount of short transcripts relative to long (Fig. 1B, lanes 2 and 3 and lanes 5 and 6). We observed the same result when using P1/P2 or P2 DNA templates. At present, the nature of the bands smaller than 83 nucleotides detected at high DNA concentration (lanes 3 and 6) is unknown. As a control, we performed the in vitro transcription/elongation assay in the presence of α-amamin, which strongly inhibits RNAPII-dependent transcription. Transcription was completely eliminated by 4 μg of α-amamin per ml, which indicates that RNA synthesis was carried out by RNAPII (data not shown). Those results indicate that premature pausing/termination of transcription complexes assembled on the c-myc promoter increases as a function of the amount of template, suggesting that a factor(s) essential for fully processive elongation may be limiting in the nuclear extract.

HIV-1 Tat Activates RNAPII Transcription Complexes Assembled on the c-myc Promoter—The block to elongation in the human c-myc gene is similar to HIV-1 transcription. Activation of HIV-1 gene expression is dependent on the presence of the viral Tat protein, which activates viral gene expression by enhancing the elongation efficiency of RNAPII complexes. This process is mediated by the interaction of Tat with its RNA target called TAR and with P-TEFb (9). We reproduced this effect in nuclear extracts of HeLa cells by transcribing a template containing the wild type HIV-1 promoter and the two G-less cassettes. In this elongation assay, exogenous recombinant Tat protein strongly increased the level of long transcripts with less effect on the level of short transcripts (Fig. 2A). Quantification of the long and short transcripts shows a bigger effect on the activation of long transcripts by Tat (Fig. 2A), which indicates an increase in the elongation efficiency of RNAPII complexes formed on the HIV-1 promoter. This activation was dependent on a functional TAR element since Tat was unable to activate an HIV-1 promoter containing a deletion in the bulge sequence (Tat binding site) of this element (Fig. 2B).

The similarities in elongation control between c-myc and HIV-1 suggest a common mechanism for the control of this phase of transcription. This predicts that cellular proteins regulating HIV elongation would function similarly for c-myc. To test this hypothesis, we cloned the HIV-1 TAR element downstream of the c-myc promoter at a position similar to its natural site in the HIV-1 gene and examined the effect of Tat in c-myc read-through transcription using the elongation assay. Transcription reactions were performed in conditions where the elongation efficiency of the RNAPII complexes formed on the c-myc promoter was low (Fig. 2C, lanes 1 and 2 and lanes 5 and 6). The addition of Tat strongly activated c-myc transcription as reflected by the preferential increase in the level of the long transcripts (Fig. 2C). Quantification of these data is also shown in graph form (Fig. 2C). Activation of transcriptional elongation
FIGURE 2. Activation of c-myc transcription by HIV-1 Tat protein. A, the transcriptional activity of Tat protein was assayed using an in vitro transcription system. Transcription reactions were carried out with a double G-less cassette containing the HIV-1 LTR and in the absence (lane 1) or presence of 50, 100, and 200 ng of recombinant Tat (lanes 2–4). Arrows indicate the position of long and short transcripts. A schematic representation of the HIV-1 LTR double G-less cassette template used in the experiment is shown on top of the panel. Quantification of the experimental data by PhosphorImager is shown in graphic form. B, TAR dependence of Tat-mediated HIV-1 transcriptional activation. The experiment described for panel A was carried out with a double G-less cassette containing a wild type (WT) or mutant (BL, bulge-less) TAR element in the absence (−) or presence (+) of 200 ng of recombinant Tat. Arrows indicate the position of long and short transcripts. C, HIV-1 Tat increases read-through transcription at sites of pausing within the c-myc gene in vitro. Transcription elongation reactions were performed with DNA templates without or with a TAR element within c-myc gene (P1/P2, P2, and P1/P2[TAR], P2[TAR], respectively) in the absence (−) and presence of 100 and 200 ng of recombinant Tat as indicated on top of the figure. Arrows indicate the migration of long and short transcripts. Experimental data from the experiment shown in this panel were quantified with phosphorimaging. D, reporter gene analysis of the activity of HIV-1 Tat in cells. DNA templates containing the HIV-1 and c-myc/TAR promoter elements driving the expression of the luciferase reporter gene were cotransfected in cells with the indicated amounts of Tat. Data are shown as the fold activation of reporter activity. The experiment was performed three times independently, and values from one representative experiment are shown.
of the c-myc gene by Tat was dependent on a functional TAR element (data not shown). Corroborating data were obtained using in vivo transient transfection experiments. The TAR-containing c-myc construct and the HIV promoter construct linked to a luciferase reporter gene were transiently transfected in cells in the absence or presence of a Tat-expressing plasmid. As shown in Fig. 2D, Tat strongly activated transcription driven by both promoters. In this transfection assay Tat failed to activate a c-myc promoter template containing a deletion in the bulge sequence of TAR (data not shown). These data demonstrate that Tat can modify the RNAPII complexes formed on the c-myc promoter to activate elongation efficiency.

In the cell-free system described here, Tat stimulates transcription by 3–10-fold, which is consistent with previous in vitro results (22, 28). In vivo studies have shown, however, that Tat increases transcription by several hundred-fold (such as the data shown in Fig. 2D). The high basal levels of HIV transcription or the inefficient re-initiation observed in vitro and the lack of an appropriate chromatin environment may account for those differences.

P-TEFb Regulates Transcriptional Elongation of c-myc in Vitro—The results of our in vitro and in vivo assays suggest that targeted recruitment of P-TEFb to the c-myc promoter by Tat/TAR increases the elongation efficiency of RNAPII complexes. If so, depletion of P-TEFb should lead to an abrogation of c-myc transcriptional activity. To test this hypothesis, we specifically immunodepleted CDK9 from HeLa cell nuclear extract. The supernatant fraction from the anti-CDK9 precipitation showed a 90% depletion of CDK9 and cyclin T1 (Fig. 3A and data not shown) but no effect on the levels of other proteins such as transcription elongation factor CA150 or splicing factor U2AF65 (Fig. 3A). The supernatant fraction from mock-depleted extract had levels of CDK9 and cyclin T1 similar to those in the original load (Fig. 3A). To evaluate the effect of the depletion on the transcriptional activity of HIV-1 and c-myc promoters, in vitro transcription elongation reactions were carried out with the supernatant fractions from the depleted extracts. Immunodepletion of CDK9 from the extract significantly reduced the level of long transcripts with minimal effect on the level of short transcripts synthesized from the HIV-1 and c-myc promoters (Fig. 3B, lanes 2 and 5, respectively). This result clearly shows the specific role of CDK9 in the elongation efficiency of RNAPII complexes formed on the HIV-1 and c-myc genes, which supports previous studies that have shown that P-TEFb is critical for RNAPII transcription elongation (29–31).

Next, we sought to test whether P-TEFb could recover the level of transcriptional elongation in the CDK9-depleted extracts. To achieve this, CDK9 and cyclin T1 were co-expressed using a recombinant baculovirus system and added to the CDK-9-depleted nuclear extract. The addition of recombinant P-TEFb to the reactions increased the elongation efficiency (Fig. 3B, lanes 3 and 6, respectively). Those results

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**FIGURE 3.** c-myc gene is regulated through a P-TEFb-dependent elongation pathway. A, immunodepletion of CDK9 from HeLa nuclear extract. Shown are the results of Western blot analysis of undepleted, mock, and CDK9-depleted nuclear extract (NE). Specific antibodies against CA150, U2AF65, CDK9, and cyclin T1 were used to localize the proteins. The positions of the proteins and the relative mobilities (in kDa) of the molecular mass markers (M) are shown on the right and left sides of the panel, respectively.

B, analysis of transcriptional elongation activity in the immunodepleted extracts. Transcription elongation reactions were performed with the indicated fractions and the HIV-1 LTR or the c-myc double G-less cassette templates. Lanes 3 and 6 show that the addition of recombinant P-TEFb (80 ng) increased elongation efficiency of both HIV-1 and c-myc transcription in the immunodepleted extracts. Arrows indicate migration of long and short transcripts.
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FIGURE 4. Analysis of PICs assembled on the HIV-1 and c-myc promoters in vitro. A, biotinylated HIV-1 and c-myc templates were incubated with nuclear extract, and PICs were purified with streptavidin-coated magnetic beads. In vitro transcription elongation reactions were performed with the purified PICs, and the transcripts were labeled with \([\alpha-\text{32P}]\text{UTP}\) and resolved by electrophoresis. Lanes 1–4 are two independent reactions performed with the HIV-1 and c-myc templates as indicated on top of the figure, respectively. Arrows indicate the migration of long and short transcripts. B, CDK9 and cyclin T1 are components of the HIV-1 and c-myc PICs. PICs were formed on the following templates: HIV-1 promoter less (HIVPless), HIV-1, c-myc promoter less (c-mycPless), c-myc, TdT, and HIV-1 with a TATA-box mutated (HIVTATAm). An association reaction with no template added was also carried out as a parallel control (−). PICs were purified with streptavidin-coated magnetic beads, and Western blot analysis of the purified PICs was then done with the specific antibodies shown at the right side of the panel. NE, nuclear extract.

FIGURE 4A shows a representative Western blot analysis of PICs using Sp1-, TBP-, cyclin T1-, CDK9-, nucleolin-, and CDK2-specific antibodies. As expected, we observed cyclin T1 and CDK9 in the PICs formed on the HIV-1 promoter. Interestingly, both proteins were also found in the PICs formed on the P2 c-myc promoter template. Proteins associated with the P-TEFb complex failed to precipitate with the promoter-less, HIV-1 TATA-box mutant, and TdT templates, which demonstrates the specificity of our assay. The absence of P-TEFb complex in the PICs formed using the TdT promoter could indicate a requirement for a recognizable TATA element to recruit this transcription complex (see below). The TdT construct was transcriptionally active in separate functional assays (data not shown; see also Fig. 6). The presence of binding of Sp1 to the TATA-box mutant template adds further specificity to the assay (Fig. 4B). Similarly, we detected only trace amounts of TBP in the PICs formed onto the TATA-box mutant and TdT templates (Fig. 4B). Sp1 was weakly detected in the c-myc PICs, which agrees with the presence of a single CT-rich functional Sp1-binding sequence upstream of the P2 promoter (18). Finally, we did not detect binding of nucleolin or CDK2 to the PICs (Fig. 4B). Those results demonstrate the presence of P-TEFb in the RNAPII complexes assembled onto the c-myc promoter in vitro and the requirement for a TATA-box sequence in P-TEFb recruitment to promoters.

To determine whether P-TEFb is recruited to the integrated HIV-1 LTR and chromosomal c-myc promoters in vivo, ChIP experiments were carried out using a Tat-inducible Jurkat cell line. This cell line simultaneously expresses the eGFP and Tat genes upon transcription activation. Induction of eGFP and Tat in PMA-treated cells was confirmed by reverse transcript-PCR and immunoblotting using anti-eGFP and anti-Tat antibodies (Fig. 5A). We could detect a weak positive signal by reverse transcription-PCR in the absence of induction (Fig. 5A), which demonstrate that those inducible systems are always a bit leaky. No signal could be detected by Western blotting analysis in the absence of PMA (Fig. 5A). Chromatin was isolated from these cells in the absence or presence of PMA treatment, immunoprecipitated with the specific antibodies, and subjected to PCR using primer sets specific for the c-myc and the HIV-1 LTR promoter regions. Fig. 5B shows the results obtained from the non-induced (Me2SO) and induced cells (PMA). As a control of the ChIP experiment, we detected RNAPII at the HIV-1 and
c-myc promoters. Importantly, we detected CDK9 at both promoters in vivo. No proteins were detected using control IgG antibodies (Fig. 5B). Control regions of an intergenic region upstream the c-myc gene or the OCT-2 gene were unaffected (Fig. 5B). Thus, we concluded that P-TEFb is recruited to both promoters in vivo.

Targeted Recruitment of CDK9 to Promoters Reveals a Strict Requirement of the TATA-box for CDK9 Activity—To further examine the role of CDK9 in transcription, we targeted CDK9 to a promoter by fusing it to a sequence-specific DNA binding domain (GAL4 1–147). We then tested the ability of the fusion protein to activate the HIV-1 and c-myc promoter elements containing appropriate GAL4-DNA binding sequences. As shown in Fig. 6B, GAL4-CDK9 effectively activated transcription when tested on the GAL4-containing HIV-1 (G5HIV-LUC) and c-myc (G5cmycP2-LUC) reporter constructs. In contrast, a catalytically inactive CDK9 protein bearing a single amino acid change (Asp-167 to Asn) in its catalytic domain. The target plasmids contained the HIV-1, TdT, and c-myc promoter sequences linked to the luciferase reporter gene. By reporter gene analysis of the activities of the indicated GAL4-CDK9 proteins on the GAL4-driven promoter-reporter constructs in HeLa cells. Expression plasmids encoding GAL4-CDK9 or GAL4-CDK9dn fusion proteins were cotransfected into HeLa cells with the indicated reporter plasmids and CMV-CAT control reporter plasmid. Cells were harvested, and activity assays were done as described under “Experimental Procedures.”

FIGURE 6. The role of promoter and proximal promoter elements in the activation of transcriptional elongation by DNA-tethered P-TEFb. A, a diagrammatic representation of the constructs used. The activator plasmid GAL4-CDK9 directed the expression of the full-length CDK9 protein fused to the GAL4 DNA binding domain. GAL4-CDK9dn expresses a kinase-inactive CDK9 protein bearing a single amino acid change (Asp-167 to Asn) in its catalytic domain. The target plasmids contained the HIV-1, TdT, and c-myc promoter sequences linked to the luciferase reporter gene. B, reporter gene analysis of the activities of the indicated GAL4-CDK9 proteins on the GAL4-driven promoter-reporter constructs in HeLa cells. Expression plasmids encoding GAL4-CDK9 or GAL4-CDK9dn fusion proteins were cotransfected into HeLa cells with the indicated reporter plasmids and CMV-CAT control reporter plasmid. Cells were harvested, and activity assays were done as described under “Experimental Procedures.” The data are presented as -fold activation relative to the GAL4 reporter sample without effector. Each histogram bar represents the mean of at least three independent transfections with at least two different preparations of each plasmid DNA.

To better understand the transcription binding site requirement for CDK9 activation, we constructed several HIV-1 and c-myc mutant reporter constructs, with GAL4 binding sites, and tested the effect of those changes for proper functioning of the GAL4-CDK9. Mutations were individually introduced into the NF-κB, Sp1, TATA-box, and E2F motifs of the HIV-1 and c-myc promoters as diagramed in Fig. 6A. Mutations of either of the two NF-κB binding sites or any of the three Sp1 binding sites or the complete ablation of NF-κB or Sp1 binding sites of the HIV-1 promoter did not impair the magnitude of GAL4-CDK9 response (Fig. 6B and data not shown). These results suggest that NF-κB and Sp1 per se are not needed for proper GAL4-CDK9 activation of the HIV-1 promoter. Interestingly, a much smaller increase in activity was seen after mutating the NF-κB and Sp1 binding sites in the c-myc promoters (Fig. 6A).
TATA-box (Fig. 6B), which indicates that this sequence was strictly required for CDK9 activity. It is important to point out that in our experimental conditions, HIV-1 TATA-mutated and wild type constructs had similar transcription levels (Table 1), which indicates that both promoters are not restricted for PIC formation, and they can be stimulated at later steps like elongation (see “Discussion”). We also explored the role of the TATA-less TdT initiator in CDK9 activation by using just the initiator or the initiator plus two NF-κB and three Sp1 binding sites (NF/Sp TdT, Fig. 6A). We found no detectable activation by GAL4-CDK9 when either TdT or NF/Sp TdT was used (Fig. 6B). This result demonstrates that a complete set of enhancer sequences is not required per se for CDK9-mediated transcription activation and that the former requires a canonical TATA-box sequence.

To explore the functional consequences of the c-myc DNA binding sites in CDK9 activation, each of the c-myc reporters drawn in Fig. 6A was cotransfected into cells together with the GAL4-CDK9 expression vector. We found that activation by CDK9 was diminished when a plasmid containing a mutation at either the Sp1 or E2F site was used. However, a more dramatic effect was observed when a mutant in the TATA-box sequence was used (Fig. 6B). Those results indicate that a strict requirement for the TATA-box element is again needed for CDK9 activation to occur. These studies indicate a critical role for this sequence element in assembling a functional transcription complex responsive to CDK9. In addition, our data indicate that the unique structure of different promoters has an important role in modulating efficiently the recruitment of elongation transcription complexes to the TATA-box. Hence, the combination of proteins bound to the TATA-box and upstream sequences provides specific surfaces to recruit elongation competent transcription complexes.

DISCUSSION

A growing body of evidence supports that control of transcriptional elongation plays a prominent role in regulating gene expression in eukaryotes (34). Efficient transcript elongation by RNAPII requires overcoming “blocks,” which is accomplished with the help of elongation factors. In addition to the widely studied c-myc and HIV-1 genes, there are many examples of genes where transcriptional pausing occurs, such as the genes for adenosine deaminase (35), tumor necrosis factor-α (36), Igk (37), c-myb (38), c-fos (39), c-mos (40), and others (13), suggesting that what happens in those genes is more the rule that the exception. Although we have gained considerable knowledge about the biochemical mechanisms underlying elongation and identified many factors involved in its regulation, several questions arise. Is there a unique mechanism of facilitating productive elongation upon pausing? What are the role of promoter context and the nature of the gene itself in transcript elongation? And are the same “set” of factors involved in this process, i.e. is the P-TEFb complex generally required to relieve RNAPII pausing?

Here we have studied the transcriptional elongation of c-myc and HIV-1 genes to gain further insights into the mechanisms of transcriptional pausing. We have used an in vitro transcription elongation system that reproduces promoter-proximal pausing of HIV-1 and c-myc genes. Our data indicate that a fraction of RNAPII complexes paused before the first HIV-1 G-less cassette since there was also an ∼3-fold increase in the intensity of the 83-nucleotide band (Fig. 2A). This is consistent with recent data that demonstrate that RNAPII complexes preferentially accumulate at approximately +41 to +45 downstream from the HIV-1 transcription site (41). This posits an intriguing question; if RNAPII pauses upstream of the 3′ end of TAR, as suggested by our results, then how does Tat stimulate HIV-1 transcription? The same question arose from the results by Zhang et al. (41). As those authors discussed, we can argue that the finding that HIV-1-infected cells accumulate short transcripts beyond +60 nucleotides in length demonstrate a low basal level transcription from the HIV-1 promoter. The incomplete block to HIV-1 transcription elongation may allow limited TAR formation and Tat expression, which could then recruit P-TEFb complex and release the paused RNAPII. Inducible factors, such as NF-κB, may contribute to the viral gene activation. As also suggested by Zhang et al. (41), an alternative mechanism is possible; Tat and P-TEFb are recruited to the HIV-1 promoter by interacting with components of the pre-initiation complex, which has been reported from several independent investigators including ourselves (42–45), and this might contribute to overcoming the block to elongation occurring downstream from the start site.

Our experiments were also designed to investigate whether P-TEFb was an essential elongation factor for the human c-myc gene. We have described a reproducible in vitro transcription/elongation assay using HeLa nuclear extracts that shows premature pausing of transcription complexes assembled on the c-myc promoter as a function of the amount of template. This suggested that a factor(s) essential for efficient transcription elongation was limiting in the extract. We suspected that this factor(s) was P-TEFb, which was first identified as a complex that was critical for overcoming an early block to transcriptional elongation (29). This hypothesis was supported by the fact that HIV-1 Tat, which depends on P-TEFb to activate, stimulates production of full-length transcripts when located at the vicinities of the start site of transcription at the c-myc promoter (Fig. 2C), a result that expands similar observations (46). We demonstrated an essential role for P-TEFb in the transcriptional elongation of c-myc gene and located CDK9 in the PICs assembled on the c-myc promoter in vitro. We have also demonstrated the binding of P-TEFb to the c-myc promoter in vivo by chromatin immunoprecipitation (Fig. 5B). This observation raises the question of how cellular promoters that are regulated by RNAPII pausing recruit P-TEFb.
Different mechanisms have been postulated for P-TEFb recruitment to transcription complexes. Some specific co-activators, DNA- and RNA-bound activators and chromatin-bound activators, have been proposed to mediate the recruitment of P-TEFb to genes (47). However, very little is known about the promoter sequences that mediate the incorporation of P-TEFb to transcription units. Our results strongly suggest that the RNAPII transcription complex that assembles on a proper TATA-box sequence mediates recruitment of P-TEFb. We have found that P-TEFb is present only in PICs formed on the HIV-1 promoter with a canonical TATA-box sequence. Taking into account that the HIV-1 core promoter region also contains an initiator element (48, 49), this result suggests that P-TEFb is recruited to the HIV-1 promoter through interactions with a TATA-box dependent and Inr-independent transcription complex. The critical requirement of the TATA-box is also suggested by the fact that P-TEFb complex is not present in PICs formed using the TdT promoter (Fig. 4B). Finally, TATA-box mutant HIV-1 and c-myc templates are unresponsive to GAL4-CDK9 activation (Fig. 6), which suggests a critical role of this sequence in assembling a functional transcription complex responsive to the activator in vivo. In the experimental system used in this work, changes in HIV-1 TATA modulated the magnitude of CDK9-induced transcription but had a much smaller influence on basal transcription (Table 1). Several authors have shown the importance of TBP for TATA-dependent and TATA-independent transcription by all three nuclear polymerases. Recruitment of TBP to regions lacking a consensus TATA box may be possible by (i) the direct binding of TBP to low affinity non-consensus binding sites in the promoter region, (ii) the tethering of TBP by upstream promoter-bound activators, (iii) the interaction of TBP with sequence-specific initiator-binding proteins, and (iv) the tethering of TBP by TBP-associated factors. In the case of the HIV-1 promoter, the core region contains a nonconventional initiator element overlapping the transcriptional start site that is essential for promoter activity (48). Adding further complexity to the LTR-driven transcription, the region between the TATA-box and the start site of transcription and the promoter-proximal element called IST (inducer of short transcripts) has also been reported to be important for efficient transcription (50, 51). It appears that the HIV-1 LTR has evolved some flexibility in tolerating alternative transcriptional initiating events for its basal transcription. This flexibility may explain why in the HIV-1 LTR the TATA-box sequence is not needed to specify the site of transcriptional initiation (52). It also may explain why the replacement of the HIV-1 TATA sequence with a randomly chosen sequence, a complete deletion of the wild type sequence, or the substitution of the TATA sequence from the SV40 early promoter or with that of the murine leukemia virus greatly reduced Tat-dependent transcriptional activation without affecting the basal promoter activity or the location of the RNA start site in some experimental systems (Refs 16 and 53 and this work). Together, those data reflect a complexity in the activity of the HIV-1 core promoter region that awaits further analysis in well defined systems. Also consistent with our data, it has been recently shown that P-TEFb stimulates transcription complex assembly through recruitment of the TBP and, surprisingly, in the absence of TBP-associated factors (54). Although the mechanism by which P-TEFb selectively recruits TBP and not TBP-associated factors remains to be elucidated, our data indicate a prominent role for the TATA-box sequence in this recruitment. It will be interesting to learn whether the c-myc promoter, which we show is regulated by CDK9, specifies transcription complexes that contain TBP but not TBP-associated factors. In our experiments, we show that alterations in the HIV-1 TATA-box reduced the amount of TBP present into the pre-initiation complexes. Similar levels of TBP were detected associated with transcription complexes assembled on immobilized TdT templates (Fig. 4). Although the role of the TATA-box as a target for TBP binding is firmly established, no studies on PIC assembly have directly monitored the effects of TATA mutations together with the removal of other HIV-1 sequences implicated in transcription. In a seminal study, Ranish et al. (55) described that deletion of the yeast HIS4 TATA-box decreased by only 2–4-fold the PIC assembly, therefore suggesting that other elements present at the promoter as well as protein–protein interactions compensate for the TATA-box mutation to permit recruitment of factors to form a stable PIC.

Our data also define the role of transcription factor binding sequences in CDK9-mediated transcriptional activation. In the case of HIV-1, targeted recruitment of CDK9 to the HIV-1 promoter activates transcription when NF-κB or Sp1 binding sequences are individually mutated. Because both sequences are required for proper P-TEFb activation (20, 56, 57), it is possible that both factors simultaneously cooperate for promoter activation. Interestingly, activation of the initiator construct by GAL4-CDK9 was not significantly improved with the inclusion of NF-κB and Sp1 binding sequences (Fig. 6), which further supports an essential role of the TATA-box in CDK9 activation. In c-myc, mutations in Sp1 and E2F binding sequences partially impaired activation, which suggests a role for those factors in assembling a functional RNAPII transcription complex responsive to GAL4-CDK9.

In summary, our findings complement current views from previous published data and establish an important role for the TATA-box sequence in assembling the P-TEFb-containing transcription complex that activates transcription. Thus, our study suggests a common mechanism of elongation control between c-myc and HIV-1 genes. Our data also indicate that upstream activator binding sequences collaborate in the recruitment of elongation-competent transcription complexes at the promoter region. We propose a common mechanism for elongation control at c-myc and HIV-1 genes with an essential role for the TATA-box and specific modulator contribution of upstream regulatory sequences, derived from the unique structure of the promoters, to form a composite surface for efficient recruitment of elongation transcriptional complexes.

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