Structure and Function of the Human Transcription Elongation Factor DSIF*

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5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DBR) is a classic inhibitor of transcription elongation by RNA polymerase II (pol II). We have previously identified and purified a novel transcription elongation factor, termed DSIF (for DRB sensitivity-inducing factor), that makes transcription5 sensitive to DRB. DSIF is composed of 160- and 14-kDa subunits, which are homologs of the Saccharomyces cerevisiae transcription factors Spt5 and Spt4. DSIF may either repress or stimulate transcription in vitro, depending on conditions, but its physiological function remains elusive. Here we characterize the structure and function of DSIF. DSIF p160, p160 is shown to be a ubiquitous nuclear protein that forms a stable complex with p14 and interacts directly with the pol II largest subunit. Mutation analysis of p160 is used to identify structural features essential for its in vitro activity and to map the domains required for its interaction with p14 and pol II. Finally, a p160 mutant that represses DSIF activity in a dominant-negative manner is identified and used to demonstrate that DSIF represses transcription from various promoters in vivo.

The nucleoside analog DBR is a classic inhibitor of transcription elongation by pol II (reviewed in Ref. 1). Although it has been used for more than three decades, its mode of action has long been a mystery. DBR is unique in that it shows no effect on transcription reconstituted with purified general transcription factors (GTFs) and pol II, whereas it potently represses transcription in crude systems or in vivo (2–4). Therefore, one or more factors apart from GTFs and pol II appear to be involved in DRB-sensitive transcription (1). Such putative factors must play general roles in pol II transcription in vivo because DBR affects most of the class II genes (4–6).

Recently, we and others have identified two elongation factors essential for DRB-sensitive transcription. One of them, positive transcription elongation factor b (P-TEFb), is a protein kinase that phosphorylates the pol II C-terminal domain (CTD) in a DRB-sensitive fashion (7–12). The CTD phosphorylation likely plays a pivotal role in pol II elongation and may be relevant to the P-TEFb function. The other, DSIF, has been purified from HeLa cell nuclear extract, based on its ability to induce DRB-sensitivity in vitro (3). DSIF is composed of 160- and 14-kDa subunits, which are homologs of the Saccharomyces cerevisiae transcription factors Spt5 and Spt4 (3, 13–15). DSIF/Spt4-Spt5 genetically and physically interacts with pol II, and thus may directly regulate pol II processivity (3, 16).

The function of DSIF in the absence of DRB remains obscure. Small amounts of DSIF, when added back to a DSIF-depleted transcription system, repress transcription only in the presence of DRB, without affecting transcription in its absence (3). Higher doses of DSIF, however, repress transcription even in the absence of DRB (3). In contrast, under limiting concentrations of NTPs, DSIF stimulates transcription elongation in the absence of DBR (3). Genetic analysis in yeast also suggests a stimulatory role for Spt4-Spt5 under low NTP concentrations in vivo (16).

DSIF p160 has many distinctive structural features (Refs. 1 and 3; see Fig. 1A). The N-terminal region is highly acidic, and several hexapeptide repeats are found at the C terminus. In the central domain, there are four stretches with significant similarity to NusG, a prokaryotic transcription termination and anti-termination factor (17–19). The corresponding region of NusG has recently been found to share homology with a class of proteins involved in translation and is termed the KOW motif, though its function is unknown (20). We have named the four stretches KOW1 to 4, respectively (see Fig. 1A).

In this report, we have characterized the structure and function of DSIF p160. We show that p160 is a nuclear protein that forms a stable complex with p14 and interacts with the pol II largest subunit. In addition, we map the regions of p160 that are involved in interactions with p14 and pol II and those that are essential for DSIF activity. Finally, we isolate a p160 mutant that acts in a dominant-negative manner and use this mutant to present evidence that DSIF is a negative regulator of transcription in vivo.

MATERIALS AND METHODS

Plasmid Constructs—For expression of recombinant proteins, the following plasmids were constructed in the expression vector pET-14b (Novagen) by subcloning from parent vectors described elsewhere (3): pET-p160Δacidic, which lacks amino acids (aa) 1–175 of p160; pET-p160Δ14BD, which lacks an 176–314 of p160; pET-p160ΔNusG, which lacks aa 314–516 of p160; and pET-p160ΔKOW1, which lacks aa 758–936 of p160.

For expression of p160ΔKOW1 and ΔKOW2, which lack aa 421–447 and aa 473–499 of p160, respectively, a polymerase chain reaction-
based overlap extension technique was employed (21). Forward and reverse mutagenic primers encompassing the deletion points were used and the amplified fragments cloned into pET-14b, generating pET-p160ΔKOW1 and pET-p160ΔKOW2. For expression of GST-p14, a fragment of pBS-DSIFp14 was subcloned into pGEX-5X-3 (Amersham Pharmacia Biotech) to generate pGEX-p14. To construct pCMV-FLAGp160 and pCMV-FLAGp160NusG, fragments of pBS-FLAGp160 and pBS-FLAGp160NusG were inserted into pCAGGS (22). To construct pCMV-HAp14, p14 full-length cDNA was inserted into pCHA, a pCAGGS derivative that contains a sequence encoding HA epitope YYDVFPDYA.

Preparation of Recombinant (r-)p16 and r-p14 Proteins—Histidine-tagged p160 and p14 were expressed in Escherichia coli strain BL21 (DE3) transformed with pET-DSIFp160 or pET-DSIFp14 (3). Cells were harvested and lysed by sonication, the lysates were cleaved by centrifugation and filtration, and r-p16 and r-p14 were purified from the supernatants by Ni-affinity column chromatography according to the instructions of the manufacturer (Novagen). To further purify the recombinant proteins, the purified fractions were subjected to SDS-PAGE, and the full-length proteins were recovered from a gel slice. The proteins were then precipitated with acetone, denatured with 6 M guanidine-HCl, and renatured by dialysis against 10 mM HEPES (pH 7.9), 10% glycerol, 50 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol as described (23, 24).

To prepare in vitro translated p160 and its derivatives, either pBS-p160, pBS-p160Teres or pBS-p160Tera was digested with appropriate restriction enzymes, some of which cleave the p160 open reading frame to produce the C-terminally truncated products. The linearized plasmids were then transcribed with either T7 or T3 RNA polymerase, followed by translation using a rabbit reticulocyte lysate system (Promega) in the presence of [35S]methionine.

p160-p14 Interaction Assays—pCMV-FLAGp160 (10 μg) and pCMV-HAp14 (10 μg) were translated into 2 × 10⁶ HeLa cells either individually or in combination. Forty-eight hours post-transfection, the cells were lysed in 500 μl of high salt buffer (50 mM Tris (pH 7.9), 500 mM NaCl, 1% Nonidet P-40) and cleared by centrifugation. The lysates were incubated with 2 μg of anti-Flag M2 monoclonal antibody (Sigma) for 2 h at 4 °C and then with 20 μl of protein G-Sepharose (Amersham Pharmacia Biotech) for 1 h at 4 °C. After washing three times with high salt buffer, the immunoprecipitates were eluted with 50 μl of SDS sample buffer and analyzed by SDS-PAGE and fluorography.

In Vitro Transcription Assays—HeLa cell nuclear extract and the phosphocellulose (P11) column fractions (P.3 and P.1.0) were prepared as described (3). The template plasmid pTF3–6C2AT, which produces a 380-nucleotide G-free transcript (3), was used. Transcription reactions were performed as described (3). In Fig. 3, 20 μl reactions containing P.1.0 (2 μl), P.3 (4 μl), r-p14 (3 ng), r-p160, or a derivative (30 ng) and pTF3–6C2AT (250 ng) were incubated for 45 min at 30 °C. Reactions were initiated by adding 2.5 μl of NTP mix (final 60 μM ATP, 600 μM CTP, 5 μM UTP, 80 μM 3'-O-methyl-GTP, and 50 units of RNase TiI) and 2.5 μl of buffer or DRB (final 50 μM). After a 10-min incubation, reactions were terminated and the G-free transcripts analyzed by 8% urea-PAGE. In Fig. 7, reactions containing HeLa nuclear extract (4 μl) and r-p160NusG mutant (0–200 ng) were incubated for the indicated times, and processed as above.

Transfection Assays—HeLa S3 cells were maintained in minimal essential medium (Nissui) supplemented with 10% fetal calf serum and l-glutamine. A total of 10 μg of DNA (5 μg of reporter plasmid, various amounts of pCMV-FLAGp160NusG, and the empty vector pCAGGS) were transfected by a standard calcium phosphate method (24) into 3 × 10⁵ cells plated on 6-cm dishes 20 h before transfection. DNA-CaPO₄ co-precipitates were removed 6 h later. The cells were harvested 48 h post-transfection, and their luciferase activities were measured. The values were normalized by protein amount and expressed as -fold activation.

RESULTS

Amino Acid Sequence of DSIF p160—In our previous study, we reported the isolation of a cDNA encoding the 160-kDa subunit of DSIF (3). The nucleotide and deduced amino acid sequences of p160 are shown in Fig. 1A. There was no ATG sequence upstream of the putative translation initiation site, and the adjacent sequence fitted the Kozak consensus sequence (25) although there was no upstream stop codon in frame. To confirm its identity, the entire cDNA was transcribed and translated in vitro, and the size of the 35S-labeled product was compared with that of p160 purified from HeLa nuclear extract and 32P-labeled by phosphorylation with casein kinase II (Fig. 1B). The phosphorylation did not change the migration of p160 (data not shown). From the indistinguishable migration of the two bands, we concluded that the cDNA encodes full-length p160.

Two groups have reported nucleotide sequences of Supt5H, a human homolog of S. cerevisiae SPT5 (26, 27). The p160 cDNA that we have cloned is almost identical to these sequences. However, the deduced amino acid sequences which we and Stachora et al. (27) report are different from that reported by Chiang et al. (26) over two large portions, probably because of sequencing errors.

p160 Is a Ubiquitously Expressed, Nuclear Protein—Northern blot analysis of p160 using mRNA from several different human tissues detected a ubiquitously expressed band of ~3.6 kilobases, a length consistent with the isolated cDNA (Fig. 2A). Because p160 possesses putative nuclear localization signals (Fig. 1A), we analyzed the subcellular localization of p160. Mammalian vectors expressing Flag-tagged p160 (Flag-p160) and HA-tagged p14 (HA-p14) were transfected into HeLa cells, and their expression was examined by immunofluorescence microscopy. p14/Supt4H is localized in the nucleus (13). As shown in Fig. 2B, Flag-p160 was also exclusively localized in the nucleus, regardless of co-expression of HA-p14. These results agree with the postulated role of p160 as a general transcription elongation factor.

p160 Forms a Stable Complex with p14—Spt5 genetically and physically interacts with Spt4 in yeast (16, 28). In addition, p160 was co-fractionated with p14 through several different columns during purification of DSIF (3). It is therefore likely that p160 associates with p14. To test this, we expressed Flag-p160 and HA-p14 in HeLa cells either individually or in combination, and immunoprecipitated them with anti-Flag antibody. As shown in Fig. 3A, HA-p14 was co-precipitated with Flag-p160 (lane 8), demonstrating their interaction in vivo.

The interaction was verified in vitro. GST-p14 affinity column or a control GST column was incubated with in vitro translated 35S-labeled p160 under various conditions. After extensive washing with the same buffer, bound p160 was eluted and analyzed. About 50% of p160 bound to the GST-p14 column, whereas no binding to the control column was detected (Fig. 3B, lanes 2 and 3). The interaction was unaffected by the presence of 0.5% Nonidet P-40 and 1 mM KC1 (lane 6). Because p14 contains a putative zinc finger motif conserved among different species (13), we examined the effect of divalent cations (lane 4) and a high concentration of a chelating agent (lane 5). Even under these conditions, p160 efficiently bound to p14, suggesting that the zinc finger is not required for the interaction.

We next mapped the region of p160 involved in p14-binding. Various forms of 35S-labeled p160 were produced in vitro, and their interactions with GST-p14 were analyzed (Fig. 3C). The C-terminal half of p160 was dispensable for the interaction (lanes 8 and 9), and fine mapping of the N terminus identified...
a minimal region of aa 176–313 sufficient for p14-binding (lane 30). Because aa 1–270 of p160 also bound to p14 equally well (lane 28), the minimal domain is aa 176–270.

Mapping the Region of p160 Important for DSIF Activity in Vitro—We next sought to identify regions important for DSIF activity. We constructed a series of p160 mutants lacking various structural motifs (see “Materials and Methods”). These mutants were expressed in E. coli and purified, and the integrity of the recombinant proteins was verified by either silver staining (Fig. 4A) or immunoblotting with an antibody raised against the extreme C terminus of p160 (data not shown).

Equal amounts (30 ng) of these mutants were assayed for DSIF activity.

**Fig. 1.** A, nucleotide and amino acid sequences of DSIF p160. The nucleotide sequence was deposited in the GenBank™/EBI Data Bank under accession number AB000516. Residues identified by sequencing purified p160 (3) are underlined. The putative nuclear localization signals are double underlined. The acidic region is indicated by an arrow, and acidic residues within this region are circled. Four stretches with similarity to the KOW motif of E. coli NusG are named KOW1 to 4 and indicated by arrows. The hexapeptide repeat sequences are indicated by open boxes. B, comparison of in vitro translation (IVT) product of the cDNA and purified p160. The entire cDNA was transcribed and translated in vitro, and the size of the [35S]-labeled product was compared with that of p160 purified from HeLa nuclear extract and [32P]-labeled by phosphorylation with casein kinase II.
was carried out using an Axiovert (Carl Zeiss). A multiple tissue Northern blot (CLONTECH) containing 2 μg/ml 35S-labeled p160 cDNA. A human β-actin probe (CLONTECH) was also used to confirm equivalent loading of intact RNA. The positions of size markers (in kilobases) are indicated at left. B, subcellular localization of p160. pCMV-FLAGp160 and pCMV-HAp14 were, either individually or in combination, transfected into HeLa cells plated onto coverslips. The nuclear extract used contains 0.01 mg/ml 4,6-diamidino-2-phenylindole (DAPI). Fluorescence microscopy was carried out using an Axiovert (Carl Zeiss).

Fig. 2. Analysis of the expression pattern of p160. A, Northern blot analysis. A multiple tissue Northern blot (CLONTECH) containing 2 μg of poly(A)+ mRNA per lane was probed for p160 expression. Northern blotting was performed using a standard technique (24) with a random-labeled 417-base pair fragment (from 1528 to 1944 nucleotides) of the p160 cDNA. A human β-actin probe (CLONTECH) was also used to confirm equivalent loading of intact RNA. The positions of size markers (in kilobases) are indicated at left. B, subcellular localization of p160. pCMV-FLAGp160 and pCMV-HAp14 were, either individually or in combination, transfected into HeLa cells plated onto coverslips. The cells were fixed with methanol-acetic acid (3:1) solution and sequentially incubated with 5% milk-phosphate-buffered saline, anti-Flag antibody, rhodamine-conjugated secondary antibody (Chemicon), and 0.01 mg/ml 4,6-diamidino-2-phenylindole (DAPI). Fluorescence microscopy was carried out using an Axiovert (Carl Zeiss).

DRB sensitivity-inducing activity (Fig. 4B). This protein amount corresponds to the “low dose” of our previous report (3). Wild type r-p160, in conjunction with r-p14, converted DRB-insensitive transcription to a process sensitive to DRB (lanes 1–4), as reported previously (3). Δrepeat similarly induced DRB-sensitivity (lanes 11 and 12). These proteins also moderately reduced the basal level of transcription, i.e., in the absence of DRB. In contrast, all other mutants had no effect (lanes 5–10 and 13–16). The slight variations seen in Δp14BD and ΔNusG were not reproducible. The acidic region, the p14-binding region, and the NusG-homology regions of p160, therefore, appear to be important for DSIF activity.

Analysis of the Interaction between p160 and pol II—p160/Spt5 physically interacts with pol II (3, 16). In addition, Spt5 genetically interacts with the two largest subunits of pol II in yeast (16). We therefore wished to determine which subunit of pol II is involved in interaction with p160. Pol II purified from HeLa cell nuclear pellet was separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, renatured, and probed with 35S-labeled p160. As shown in Fig. 5A, a clear band of ~200 kDa, which co-migrates with the largest subunit of pol II, was detected. p160 also reacted with GST-p14 protein which was similarly blotted onto the membrane. From these results, we conclude that p160 interacts with pol II through its largest subunit pol IIa.

We next mapped the region of p160 involved in pol II binding. Various forms of p160 were synthesized in vitro, and incubated with purified pol II and anti-CTD antibody. The immunocomplexes were precipitated, and the presence of p160 was analyzed. As shown in Fig. 5B, the C-terminal deletion of p160 did not affect the interaction (lanes 1–8). One predicted role for the KOW motif is interaction with RNA polymerases (20). Fine mapping, however, revealed that aa 313–420 of p160, an N-terminal region in close proximity to the KOW motifs, is sufficient for pol II binding (lane 23). aa 420–757 of p160, which contains all four of the KOW motifs, also bound to pol II weakly (lane 24). Results of deletion analyses described above are summarized in Fig. 6.

Identification of a p160 Mutant That Represses DSIF Activity in a Dominant-Negative Manner in Vivo—Because DRB is a nonphysiological compound, it is important to address the role of DSIF in the absence of DRB. Functional analyses of DSIF to date have been limited to in vitro experiments, and there is no evidence regarding its role in vivo. As a first step, we sought to isolate a dominant-negative mutant of p160. We speculated that p160 mutants that complex with p14, but have no DSIF activity, would act in a dominant-negative manner. We employed one such mutant, ΔNusG, and tested its dominant-negative effect in vitro. Increasing amounts of the mutant protein were incubated with HeLa nuclear extract for 45 min, and then transcription reactions were carried out in the presence or absence of DRB (Fig. 7A). Addition of 200 ng of the mutant slightly reduced DRB-sensitivity of the nuclear extract (lanes 7 and 8). Furthermore, when the preincubation time was prolonged to 180 min, DRB sensitivity was markedly reduced (Fig. 7B, lanes 11 and 12). The nuclear extract used contains ~30 ng of endogenous p160 protein (3). Thus, 6- to 7-fold excess of the p160 mutant protein, and a 3-h incubation period, were sufficient to inactivate most of the endogenous DSIF. It is likely that during this period, endogenous wild type p160 is replaced by the mutant to form an inactive DSIF complex with p14. These results also suggest that the p160 mutant is inactive, not simply because of incorrect folding, but because a specific function of p160 encoded within the deleted region has been lost.

DSIF Represses Transcription from Various Promoters in Vivo—Next, we used ΔNusG to analyze the effect of DSIF on transcription in vivo. Four different reporter plasmids were used in which expression of the luciferase gene is controlled by the adenovirus E4 promoter, the HIV-1 long terminal repeat (Fig. 8A, LTR), the rat somatostatin (som.) promoter, and the mouse metallothionein (metal.) promoter. These promoters possess a typical TATA box but no “initiator” consensus sequence and are subject to regulation by transactivators of different classes. The E4 promoter is activated by ATF and GABP (23, 29); the HIV-1 long terminal repeat is activated by NF-κB, Sp1, and Tat (30); the somatostatin promoter is stimulated by the c-AMP signaling pathway and CREB (31); and the metallothionein promoter is induced by heavy metal and MTF-1 (32).
One of these reporter plasmids (5 μg) was co-transfected with increasing amounts of a plasmid expressing ΔNuSG into HeLa cells. The total amount of DNA added per transfection was adjusted to 10 μg with the empty vector pCAGGS. ΔNuSG stimulated luciferase expression 5- to 10-fold in a dose-dependent manner, irrespective of the promoter used (Fig. 8A). The same transfectants were examined for expression of the ΔNuSG protein. Immunoblotting with anti-ΔNuSG antibody detected the band of ΔNuSG just below the endogenous ΔNuSG (Fig. 8B). Provided that the reporter and the effector plasmids were introduced and expressed in 30% of the total cells, as estimated by immunostaining (not shown), approximately 1-, 3-, and 10-fold excesses of the mutant protein were obtained (lanes 2–4). These levels are consistent with that at which the dominant-negative effect is observed in vitro (Fig. 7). Taken together, these results indicate that DSIF represses transcription from these promoters in vivo, and reduction of the endogenous DSIF activity leads to derepressed transcription.

**DISCUSSION**

Structure of DSIF p160—Results of deletion analyses are summarized in Fig. 6. Functions of p160 to interact with p14 and pol II were assigned to different domains between the acidic region and the KOW motifs. p160-p14 interaction was shown to be very stable, remaining unaffected by 1M KCl or 0.5% Nonidet P-40 (Fig. 3B). However, we could not find any protein-protein interaction motifs within the p14-binding domain. Because this region is relatively hydrophobic (Fig. 6, top), hydrophobic interactions may contribute to the stable interaction between p160 and p14. This interaction seems dis-
pensable for p160-pol II interaction because p160 mutants lacking the p14-binding domain can still bind to pol II (Fig. 5).

The C-terminal repeat sequences were dispensable for DSIF activity in vitro (Fig. 4). Analysis in yeast, however, has demonstrated that deletion of the C-terminal part of SPT5 impairs complementation of Spt− phenotype (15). It is therefore possible that the C-terminal repeats play some regulatory roles in vivo. Interestingly, this portion is very rich in Ser, Thr, and Tyr and has many phosphorylation sites for several protein kinases. Thus, DSIF activity may be regulated by phosphorylation in vivo (27). On the other hand, most of the N terminus was required for DSIF activity. Specifically, deletion of either the acidic region, the p14-binding domain, or one of the two N-terminal KOW motifs abolished p160 function. At present, however, we could not assign functions to the acidic region or the KOW motifs. These elements may be involved in interactions with unidentified partners.

Negative Versus Positive Role of DSIF—In this study, we demonstrated that DSIF acts as a negative regulator, at least on some promoters, in vivo.

SPT4 and SPT5, yeast counterparts of the DSIF subunits, have been identified as extragenic suppressors of δ insertion mutations of HIS4 or LYS2 genes (14, 15, 33; reviewed in Ref. 34). When the δ sequence is inserted in the upstream region of a gene, the transcription signal directs transcription from the δ promoter and interferes with normal transcription of the adjacent gene. Another mutation in SPT4 or SPT5 gene suppresses the aberrant transcription, restoring transcription from the normal site. A possible interpretation of this result is that Spt4-Spt5 up-regulates transcription from the δ, and down-regulates transcription of the adjacent gene. We therefore do not discount the possibility that DSIF also functions as a pos-
activity in a dominant-negative manner in vitro. A, and B, indicated amounts of p160\(\Delta NusG\) protein were incubated with HeLa nuclear extract (NE) and template for the indicated times, and transcription reactions were carried out as shown at the top. The transcripts were quantified using a BAS image analyzer (Fuji). The relative inhibition by DRB was calculated and shown below.

We therefore used a dominant-negative mutant of p160 to probe the function of DSIF. Thus, data obtained from overexpression of SPT5 using a 2\(\mu\) plasmid leads to Spt activity. This indicates that the endogenous DSIF generally represses transcription from the reporter gene; if the endogenous DSIF influenced only part, say 10\%, of the transcription, the dominant-negative effect would not be observed. This is consistent with the idea that DSIF generally affects transcription of the class II genes, as expected from its involvement in DRB action.

DSIF subunits are also implicated in other cellular processes such as chromatin structure, cell cycle, and HIV replication (27, 36; reviewed in Refs. 1 and 34). The dominant-negative mutant of p160 isolated here would be a useful tool to examine possible DSIF involvement in these events.

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