Human SSRP1 Has Spt16-dependent and -independent Roles in Gene Transcription*

Yanping Li, Shelya X. Zeng, Igor Landais, and Hua Lu

From the Department of Biochemistry and Molecular Biology, Oregon Health & Science University, Portland, Oregon 97239

The facilitating chromatin transcription (FACT) complex, a heterodimer of SSRP1 and Spt16, has been shown to regulate transcription elongation through a chromatin template in vitro and on specific genes in cells. However, its global role in transcription regulation in human cells remains largely elusive. We conducted spotted microarray analyses using arrays harboring 8308 human genes to assess the gene expression profile after knocking down SSRP1 or Spt16 levels in human non-small cell lung carcinoma (H1299) cells. Although the changes of these transcripts were surprisingly subtle, there were ~170 genes whose transcript levels were either reduced or induced >1.5-fold. Approximately 106 genes with >1.2-fold change at the level of transcripts were the common targets of both SSRP1 and Spt16 (~1.3%). A subset of genes was regulated by SSRP1 independent of Spt16. Further analyses of some of these genes not only verified this observation but also identified the serum-responsive gene, egr1, as a novel target for both SSRP1 and Spt16. We further showed that SSRP1 and Spt16 are important for the progression of elongation RNA pol II on the egr1 gene. These results suggest that SSRP1 has Spt16-dependent and -independent roles in regulating gene transcription in human cells.

In eukaryotic cells, DNA is packaged with core histones and other chromosomal proteins in the form of chromatin, which limits the accessibility of DNA and inhibits the progression of RNA polymerases as they copy genetic information from the DNA. Thus altering the repressive nature of chromatin is necessary for the cells to implement all of the nuclear activities on chromatin (1). There are at least three types of protein complexes for this function (1, 2). The first type acts by covalently modifying the histones and non-histone chromatin proteins through phosphorylation, acetylation, ubiquitylation, and/or methylation (1, 2). The second type of complex uses ATP hydrolysis to mobilize and/or to alter the structure of nucleosomes, such as the SWI/SNF complex (2–5). The third type of chromatin-modulating complex disrupts and deposits the nucleosomes without utilizing ATP during transcriptional elongation (4, 6). One of the latter members is facilitating chromatin transcription (FACT)2 (4, 6, 7).

FACT is a heterodimeric complex consisting of Spt16 and SSRP1 (structure-specific recognition protein-1) (8, 9). Both Spt16 and SSRP1 are highly conserved in all eukaryotes. Human Spt16 is a 120-kDa protein and contains a highly acidic and serine-rich carboxyl terminus. It binds to H2A-H2B dimers and to mononucleosomes (10). It has 36% identity to its Saccharomyces cerevisiae ortholog Spt16/Cdc68 (10). The yeast Spt16/Cdc68 was identified in two independent screens for genes involved in transcription regulation (11, 12). Genetic studies in yeast suggest that Spt16/Cdc68 is required for the normal transcription of many loci (11) and has both positive and negative effects on gene expression (12). Spt16/Cdc68 is essential for yeast cell growth because spt16 null mutants are nonviable (11). The mechanism of how Spt16/Cdc68 affects transcription is suggested by the placement of Spt16/Cdc68 into a histone group of spt (suppressor of Ty) genes that encode histones and also functionally related proteins, including Spt4, Spt5, Spt6, Spt11 and Spt12 (11). This group of proteins functions by altering chromatin properties and increasing or decreasing their dosage affects on transcription regulation (13). The partner of Spt16 in the FACT complex, SSRP1, is a high mobility group (HMG) domain containing protein. It binds to cruciform or linear duplex DNA as well as DNA modified by the anticancer drug cisplatin (8, 14–16). The conserved amino terminus of SSRP1 is homologous to the yeast Pob3 protein, whereas the function of the HMG domain is provided by the small HMG-box polypeptide Nhp6 in yeast (17–19). The human SSRP1 counterparts of the yeast Pob3 and Nhp6 proteins were detected during apoptosis as the products of caspases 3- and 7-mediated cleavage of SSRP1 (20), indicating the importance of SSRP1 for cell survival. Indeed, both yeast Pob3 and mammalian SSRP1 are essential for cell (17, 18, 21) and animal (22) viability. Similar to Spt16, SSRP1 also has an acidic and serine-rich carboxyl terminus that most likely facilitates its binding to histone proteins. Supporting this is the observation that SSRP1 can bind to H3-H4 tetramers (10). The current model proposes that FACT disrupts nucleosomes, which allow RNA polymerases to access DNA, and then it reassembles the nucleosomes (10, 21). This property gives the

* This work was supported by NCI Grants CA93614, CA095441, and CA079721 from the National Institutes of Health (to H. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology/L224, Oregon Health & Science University, 3181 SW Sam Jackson Park Rd., Portland, OR 97239. Tel.: 503-494-7414; E-mail: luh@ohsu.edu.

2 The abbreviations used are: FACT, facilitating chromatin transcription; CHIP, chromatin immunoprecipitation; RT, reverse transcription; HMG, high mobility group; CTD, carboxy-terminal domain; pol, polymerase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; SRF, serum-response factor; sRNA, short interfering RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PIC, preassembled initiation complex; R, reverse; F, forward; tet, tetracycline.
FACT complex the ability to regulate transcription initiation (23, 24), elongation (7, 24, 25), and DNA replication (17, 26, 27) and also to be involved in DNA damage response (28, 29). In addition to its general role, SSRP1 also functions as a co-regulator for several transcription activators, such as serum-response factor (SRF) (30) and p63 (31). Despite the current knowledge, it remains obscure if SSRP1 has an Spt16-independent role in gene regulation. Also, it is still unclear if FACT plays a global or gene-specific role in transcriptional regulation in human cells.

To address these questions, we generated tet-inducible siRNA cell lines for each of these two proteins using H1299 cells that are p53-deficient (31). Using these cell lines, we performed spotted microarray analysis and found that the expression of many genes was altered after ablation of the endogenous SSRP1 or Spt16 levels by siRNA. Surprisingly, the effect was moderate. However, there was a subset of genes (~170) whose expression was either up-regulated or down-regulated after induction of siRNA against SSRP1 or Spt16. We further characterized some of the genes that displayed more apparent changes and found that SSRP1 and Spt16 shared common targets, as well as individually regulated genes. In particular, we identified the serum-responsive gene, egr1 (early growth response 1), as a novel target for both SSRP1 and Spt16. Either SSRP1 or Spt16 was indispensable for the expression of EGR1 in response to serum stimulation. We further elucidated that SSRP1 and Spt16 are important for the progression of RNA pol II on the coding region of the egr1 gene. Hence, our study suggests that SSRP1 and Spt16 indeed work together for the expression of a number of genes, whereas SSRP1 also appears to have an independent role in regulating the expression of a subset of genes in human cells.

MATERIALS AND METHODS

Plasmids and Antibodies—The pHetto siRNA cloning vector was the generous gift of Mathew Thayer and Dan Stauffer (Oregon Health & Science University, Portland, OR) (32). Oligonucleotides ctagGCTCAGACTGCTACCtccaa-gagGGGTAGAGCAGGTCTGAGCtttttggaaa and agcttttc-caaaagGGTTAGAGCAGGTCTGAGCtccaa-gagGGGTAGAGCAGGTCTGAGCtttttggaaa were synthesized (Dharmacon). pcDNA3-FLAG-SSRP1 was described previously (31). Anti-SSRP1 and anti-Spt16 antibodies were used for Western blot assays, as described previously (31, 33). Mouse monoclonal SSRP1 antibody 5B10 was generated by Zymed Laboratories Inc. and purified as described previously (20). The anti-EGRI, anti-SRF, and anti-Spt16 (used in chromatin immunoprecipitation assay) antibodies were purchased from Santa Cruz Biotechnology. The mouse monoclonal RNA polymerase II H5 antibody, which recognizes the RNA pol II Ser-2-phospho-isofom, was purchased from Babco-Covance. The anti-FLAG, anti-α-tubulin, rabbit polyclonal IgG, mouse monoclonal IgG, and mouse monoclonal IgM antibodies were purchased from Sigma.

Cell Culture—H1299 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 0.1 mg/ml streptomycin, at 37 °C in 5% CO2.

Generation of Inducible Tet-On Cell Line—H1299pcDNA6-TR cells, which stably express the tet repressor, were transfected with pHettoScramble, pHettoSSRP1siRNA, or pHettoSpt16siRNA plasmid and then selected in the medium containing 90 μg/ml hygromycin. Individual colonies were expanded into 12-well plates. To induce the siRNA expression, doxycycline was added to the media at a final concentration of 5 μg/ml. After doxycycline induction, cells were harvested for cell lysis preparation. SSRP1 or Spt16 expression level was checked by Western blot with anti-SSRP1 or anti-Spt16 antibodies. The colonies, which express significantly reduced levels of SSRP1 or Spt16, were maintained and used for further study.

Spotted Microarray—One clone for each cell line (clone 19 of H1299pHettoSSRP1siRNA and clone 20 of H1299pHetto-Spt16siRNA) was used in the spotted microarray experiment. RNA for the Tet-On (doxycycline treatment) samples and the Tet-Off (no doxycycline treatment) samples were prepared from three independent experiments using the Qiagen RNeasy mini kit. RNAs prepared from the SSRP1 and Spt16 siRNA Tet-Off samples were pooled as a control to compare between SSRP1siRNA and Spt16siRNA samples. The samples were sent to the Microarray Core at Oregon Health & Science University.

The SMChumC8400A array was used in the experiment. All samples were amplified using linear T7 amplification (MessageAmp, Ambion) and examined for integrity using a BioAnalyzer (Agilent). Reverse transcription was used to synthesize a cDNA containing aminomethyl-modified dUTP (Cy3cribe Post-labeling; Amersham Biosciences). Using aminomethyl-modified dUTP in both strands eliminates the requirement for dye swap experiments. Aminomethyl-modified cDNA was incubated with Cy-dye esters for a nonenzymatic and covalent attachment of either Cy5 or Cy3 to the cDNA. The experimental sample was labeled with Cy5, and the control sample was labeled with Cy3. Following cleanup, selected Cy5 and Cy3 targets were combined and applied to each of two identical slides. Arrays were hybridized using M-series LifterSlips (Erie Scientific) and deep well hybridization chambers (TeleChem). Hybridized arrays were scanned on a ScanArray 4000 XL (PerkinElmer Life Sciences) using ScanArray Express software, and ImAge (BioDiscovery) was used to extract data from the image.

The resulting data file was loaded into GeneSight for normalization using intensity-based local regression (Lowess). The normalized data were used for further segregation and clustering as described in the figure legend.

RT-PCR—Total RNA was isolated from cells after different treatments, using the TRizol (Invitrogen) protocol or Qiagen RNeasy mini kit. Reverse transcription of 5 μg of total RNA was performed in a 20-μl reaction using SuperscriptII reverse transcriptase (Invitrogen) reagent, dNTP, and oligo(dT)15 primer. After reverse transcription, 30 μl of diethyl pyrocarbonate H2O was added to the reaction. 1 μl of reverse transcription reaction
Transcription Regulation by SSRP1 and Spt16

was used in the following PCRs with the following primers: egr1 F, 5’-CTTCTCCTCTACTGGACCCAC-3’, and R, 5’-GGACTGCACTGCTTGATG-3’; plau F, 5’-ATCAGCCAGCTGTGACTGAG-3’; GACTGTTTTCTCTCGGCTC-3’, and R, 5’-TCTGACCGGAGCTGGGTTTTCCTG-3’; id2 F, 5’-CTCAGGGTAGGTTCTCGGGACT-3’; and β-actin F, 5’-ATCAGCCAGGTGGGTTTTCCTG-3’.

Real Time PCR—1 μl of RT reaction was used in the real time PCR. A 15-μl reaction was performed using the SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacturer’s protocol, and amplified on the ABI7900HT. Threshold cycles (Ct) for three replicate reactions were determined using SDS2. The relative transcript abundance was calculated following normalization with the GAPDH amplon. The data for p21 and EGR1 were collected at 80 °C, and the data for other target genes were collected at 83 °C. The following primers were used: egr1 F, 5’-GGAAGCCTGGAGGACTCAG-3’, and R, 5’-GGAGACTGCACTGCTTGATG-3’; plau F, 5’-ATGCTGATGGAGGTGC-3’; and R, 5’-TCTGACCGGAGCTGGGTTTTCCTG-3’; id2 F, 5’-TCTGACCGGAGCTGGGTTTTCCTG-3’, and R, 5’-TTACTCTCATGGAACTGCTGTTG-3’; spf16 F, 5’-AGATATGGAAGCTGTAACG-3’, and R, 5’-GCCTACTCTCGTGGCTGATG-3’, and R, 5’-GCCTACTCTCGTGGCTGATG-3’.

Transient Transfection—H1299 cells (60% confluence in 60-mm plates) were transfected with 3 μg of pcDNA3 or pcDNA3-FLAG-SSRP1 using TransFectin (Bio-Rad). Total RNA was extracted after 48 h of transfection.

siRNA Transient Transfection and Serum Stimulation Assays—H1299 cells (60% confluence in 60-mm plates) were transfected with 30 nM of the scramble, SSRP1siRNA, or Spf16siRNA using SiLentFect (Bio-Rad). At the same time, DMEM containing 10% FBS was changed to DMEM containing 0.25% FBS for serum starvation. After 48 h, cells were cultured in DMEM with 20% FBS for serum stimulation. The cells were harvested at different time points for Western blotting or RNA extraction. For Western blotting, the cell lysates were prepared as described (31), and 30 μg of cell lysates were used.

Cell Growth Assays—H1299pHteto-SSRP1siRNA, and H1299pHtetoSpf16siRNA inducible cell lines were seeded at 4 × 10^4 cells/60-mm plate and induced for siRNA expression by adding 5 μg/ml doxycycline to the media. After 4 days of induction, the cell number was counted, and viable cells were compared among the scrambled siRNA-, SSRP1siRNA- and Spf16siRNA-expressing cells.

Chromatin Immunoprecipitation (ChiP)-Real Time PCR—H1299 cells were cultured in DMEM containing 0.25% FBS for serum starvation. After 48 h, cells were switched to media containing 20% FBS and harvested at 0, 5, and 30 min post-serum stimulation. ChiP assays were carried out as described previously (31, 34) with the indicated antibodies. After reverse of cross-linking, DNA was purified by miniprep kit (Qiagen) and eluted in 50 μl of elution buffer. 1 μl of ChiP DNA or input DNA was used as templates in real time PCRs. A 20-μl reaction was performed using the SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacturer’s protocol, and amplified on the ABI7300. Threshold cycles (Ct) for three replicate reactions were determined using the 7300 system SDS software. The relative fold change among the ChiP DNA samples was calculated following normalization with the input DNA. The following primers were used in the real time PCR: primers for negative control, egr1 upstream 5990F, 5’-CACGGGCTGAGCTGTCG-3’, and egr1 upstream 5839R, 5’-AGAAAAGCGGATTCCGATATCC-3’; primers for promoter region, egr1 upstream 440F, 5’-CCCGGGAATTCGACATATTAGGAGC-3’, and egr1 upstream 2919R, 5’-AGTTCCGGGTTCGCCGCACTG-3’.

RESULTS

The Establishment of siRNA-inducible Cell Lines—To determine whether SSRP1 and Spf16 are required for global or genespecific transcription in human cells, we established inducible SSRP1 or Spf16 siRNA tet H1299 cell lines. In the presence of doxycycline, the expression of siRNA can be induced to downregulate its target mRNA. Indeed, as shown in Fig. 1, both the protein and the mRNA levels of either SSRP1 or Spf16 were markedly knocked down when the cells were cultured with...
Transcription Regulation by SSRP1 and Spt16

The list of the 12 specific genes whose transcript levels were differentially regulated in the SSRP1 or Spt16 siRNA samples, as identified in supplemental Fig. S1A.

| GenBank™ | Ave_Spt16_FC | Ave_SSRP1_FC | Symbol | Gene name |
|----------|--------------|--------------|--------|-----------|
| R70601   | 1.25949      | −2.4014      | Unknown protein | |
| N78083   | 1.21344      | −1.5576      | Unknown protein | |
| R22977   | 1.24888      | −1.5241      | MSN | Moesin |
| AA232856 | 1.21156      | −1.3845      | TOPI | Topoisomerase (DNA) I |
| H00817   | 1.24823      | −1.2726      | LYP1A | Lykophosterophase 1 |
| AA6620528| −1.2894      | 1.268        | Unknown protein | |
| AA701502 | −1.2371      | 1.25759      | PDGFA | Platelet-derived growth factor α polypeptide |
| AA676458 | −1.2318      | 1.30321      | LOX | Lysyl oxidase-like 2 |
| R08261   | −1.3456      | 1.45108      | Unknown protein | |
| AA610040 | −1.3499      | 1.45112      | HIST1H2BA | Histone 1, H2ba |
| AA653105 | −1.3104      | 1.51582      | HIST1H2AC | Histone 1, H2ac |
| AA598517 | −1.4432      | 1.82049      | KRT8 | Keratin 8 |

The alteration of 8308 genes was compared between the SSRP1 and Spt16 siRNA samples (three samples per cell line). The genes with more than a 1.2-fold change in both SSRP1 siRNA and Spt16 siRNA samples were displayed by unsupervised hierarchical clustering. This analysis revealed that ~118 genes were either up-regulated or down-regulated with >1.2-fold change in both of the SSRP1 and Spt16 siRNA samples (supplemental Fig. S1A). Most of the genes (106 genes) appeared to be the common targets for both SSRP1 and Spt16, suggesting that the regulation of these genes may be executed by the FACT complex. There were more down-regulated genes (73 or 75 genes) than up-regulated genes (45 or 43 genes) in the SSRP1 or Spt16 siRNA samples. These results suggest that SSRP1 and Spt16 work together to enhance the expression of most of their target genes, although they may also act to repress the expression of a subset of genes in human cells. This result is consistent with previous results in yeast (12). However, the 118 genes should not be the final number of SSRP1 and Spt16 targets in human cells because the cDNA array used for our study only contained 8308 genes. For example, p21, a previously identified target for SSRP1 (31), was not in this array. Even with this limited number of genes in the array, our gene expression profile data suggest that FACT may not play a global role in gene transcription, as only ~1.3% of the tested genes (106 of 8308 genes) displayed similar changes at their transcript levels in both SSRP1 and Spt16 knockout samples (supplemental Fig. S1A).

To further analyze the 118 affected genes, we classified them into 10 different groups based on their functions in biological processes. As shown in supplemental Fig. S1B, they are involved in a broad spectrum of functions, including cell growth/maintenance (23 or 27 of 118), nucleic acid metabolism (19 or 21 of 118), signal transduction (8 or 9 of 118), protein metabolism (11 of 118), biosynthesis (3 of 118), cell adhesion (3 of 118), etc. Most of the SSRP1 and Spt16 target genes (73 or 81 of 118) encode either novel proteins with unknown functions or proteins with unclassified functions or hypothetical proteins, and thus are put into the unclassified group (supplemental Fig. S1B). These results indicate that SSRP1 and Spt16 have a relatively broad role in various cellular activities. However, a majority of them are involved in cell growth and/or maintenance and metabolism, which are essential for cell growth. These data support the notion that SSRP1 and Spt16 are essential for cell viability (17, 18, 21). Indeed, ablation of either SSRP1 or Spt16 by inducible siRNA in H1299 cells severely reduced the number of viable cells (supplemental Fig. S1D). This result was also repeated in 293 cells (data not shown). It was surprising that the changes of gene expression were no more than 4-fold after knockdown of SSRP1 or Spt16 (supplemental Fig. S1, A and C; Tables 1–3). These moderate changes could be due to two possibilities. 1) SSRP1 or Spt16 might act as a cofactor in cells, so reduction of their levels would not drastically affect the gene expression profile; or 2) knockdown of SSRP1 or Spt16 was not 100%, so the residual protein might be still sufficient for transcription regulation, although to a lesser extent. Nevertheless, our results suggest that the FACT complex is important for up-regulating or down-regulating gene-specific transcription, rather than for global gene regulation. This notion was also confirmed in SSRP1 siRNA expressing 293 cells using cDNA microarray analysis, although with some variations in terms of specific target genes (data not shown).

SSRP1 and Spt16 May Have Independent Roles in Regulating the Expression of a Subset of Genes—In addition to sharing a common set of target genes (supplemental Fig. S1A), SSRP1 and Spt16 differentially regulated a subset of genes. As shown in the middle part of supplemental Fig. S1A, and also in Table 1, 12 of the 118 target genes were differentially regulated by SSRP1 and Spt16. These data suggest that SSRP1 and Spt16 may also have independent roles in gene regulation. Because the analysis for supplemental Fig. S1A discarded the data that showed changes in one (such as the SSRP1-knockdown), but not in the other (such as Spt16-knockdown), this approach would overlook what might be significant in either one of the SSRP1- and Spt16-knockdown samples. To avoid this bias and to identify...
more potential target genes for either SSRP1 or Spt16 in all the samples, we also analyzed all of the genes whose expression either increased or decreased >1.5-fold with a 95% confidence interval in at least one set of siRNA samples. As shown in supplemental Fig. S1C, 171 genes were identified among the six samples. Among these genes, some were down-regulated in the SSRP1 siRNA samples, compared with the Spt16 siRNA samples, and another set of genes was up-regulated in SSRP1 siRNA samples but down-regulated in Spt16 siRNA samples. Although there were some variations among the triplicates per each siRNA line, most of the transcript levels were reproducible overall within one siRNA cell line (supplemental Fig. S1C).

To analyze the data further, we picked up the top five genes whose expression was more apparently up- or down-regulated in either SSRP1 siRNA samples or Spt16 siRNA samples, as listed in Table 2 and Table 3. As expected and discussed above, some of them appeared to be common targets for both SSRP1 and Spt16, such as *egr1*, *rpc32*, and *cpne6* (neuronal), *id2* (inhibitor of DNA binding 2, dominant negative helix-loop-helix protein), *plau* (Plasminogen activator, urokinase), and *krtr8*. These results suggest that SSRP1 and Spt16, besides their common roles in regulating the expression of certain targets, may also have independent roles in gene regulation in human cells.

**SSRP1 Has an Spt16-independent Role in Gene Transcription**—As revealed in Table 2, knocking down SSRP1 led to the decrease of *egr1*, *plau*, and *dusp5* transcripts, but it also resulted in the increase of *id2* transcripts. Among them, only *egr1* appeared to require Spt16, as Spt16 knockdown did not cause apparent changes of *plau*, *dusp5*, and *id2* transcripts. These results suggest that SSRP1 may have Spt16-independent roles in regulating the expression of a subset of genes. To verify this possibility, we performed a series of RT-PCR and real time PCR analyses for these four genes using the same cell lines, as shown in Fig. 1. Indeed, we reproduced the microarray results. As shown in Fig. 2, the expression of *dusp5*, *plau*, and *id2* was altered in the presence of SSRP1 siRNA but not of Spt16 siRNA, whereas the transcription of *egr1* was inhibited when either SSRP1 siRNA or Spt16 siRNA was induced. In addition, the expression of *p21*, a previously identified target for SSRP1 (31), was markedly reduced when SSRP1, but not Spt16, was knocked down by siRNA (Fig. 2), verifying our previous study (31). These effects were p53-independent, as H1299 cells are p53-deficient.

To exclude the possibility that the above results were tet cell line-specific, we transiently introduced oligomers of siRNA into H1299 cells and tested the expression of *egr1*, *plau*, and *id2* using a real time PCR assay. As shown in Fig. 3A, transient transfection of either SSRP1 or Spt16 siRNA significantly reduced the level of SSRP1 or Spt16. Also, the alterations of the expression of the three target genes were consistent with the result in Fig. 2. This result was also repeated in 293 cells (data not shown).

To determine whether overexpression of SSRP1 may have the opposite effect on the expression of the four potential target genes, we transiently introduced exogenous FLAG-SSRP1 into H1299 cells. The mRNA levels of *egr1*, *dusp5*, *plau*, and *id2*...
were assessed using RT-PCR. Consistent with the results in Figs. 2 and 3A, overexpression of FLAG-SSRP1 induced the transcription of egr1, dusp5, and plau but reduced the transcription of id2 (Fig. 3B). These results suggest that SSRP1 can up- and down-regulate the expression of a subset of genes, some of which may be regulated independently of Spt16, although it remains to be determined whether this regulation is at the initiation or elongation step during transcription.

The Expression of EGR1 in Response to Serum Stimulation Requires Both SSRP1 and Spt16—The fold change of these target genes was moderate, suggesting that SSRP1 and Spt16 may work as a cofactor of transcriptional activators or repressors in human cells. One question was whether SSRP1 requires Spt16...
for its regulatory function in transcription. Interestingly, egr1, one of our identified target genes, as described above, appeared to require both of these proteins for expression. Also, interestingly, the egr1 gene contains six serum-response elements (CARG-box) in its promoter region (38). The CARG-box is the binding site of SRF (39), for which SSRP1 has been previously identified as a co-activator (30). Thus the egr1 gene serves as a proper target to address the above specific question.

EGR1 is a zinc finger containing transcription factor (40). It belongs to the cellular immediate early genes that rapidly respond to serum stimulation (40). To test whether SSRP1 is required in serum-induced EGR1 expression, we conducted a set of serum stimulation experiments. Under normal or serum starvation conditions, the EGR1 protein level was detected at extremely low levels (Fig. 4A). After 20% serum stimulation for 1.5 h, the EGR1 level was significantly increased. This induction was markedly inhibited when the cell was transiently transfected with SSRP1 siRNA before serum stimulation (Fig. 4A). This result demonstrates that SSRP1 is required for the EGR1 induction in response to serum stimulation.

The egr1 gene was also identified as a potential target for Spt16 (Table 2). To investigate whether Spt16 is also required in the EGR1 induction in response to serum stimulation, we analyzed the effect of Spt16 siRNA on the EGR1 expression after serum stimulation. The induction of EGR1 was inhibited at both the RNA (Fig. 4C) and the protein level (Fig. 4B) when Spt16 was knocked down. To determine whether overexpression of SSRP1 can rescue this inhibition, we introduced FLAG-SSRP1 into the Spt16 siRNA expressing cells and found that even overexpression of exogenous FLAG-SSRP1 could not rescue the inhibitory effect of Spt16 siRNA on the induction of EGR1 in response to serum stimulation (Fig. 4, B and C). Taken together, these results demonstrate that both Spt16 and SSRP1 are indispensable for the regulation of serum-responsive EGR1 expression.

To distinguish if the inhibition of SSRP1 and Spt16 siRNA on EGR1 expression is because of the inhibition of the EGR1 expression level or the delay of EGR1 expression during the serum stimulation course, we performed a double SSRP1 and Spt16 siRNA treatment to investigate the effect on the EGR1 expression.
expression after serum stimulation at different time points. As shown in Fig. 4D, EGR1 expression after serum stimulation displayed similar kinetics between scrambled siRNA-treated cells and double SSRP1 and Spt6 siRNA-treated cells, but its level was markedly reduced in the SSRP1 and Spt6 siRNA-treated cells. These data suggest that SSRP1 and Spt6 regulate the expression level of EGR1 and not the onset of EGR1 expression during serum stimulation.

SSRP1 and Spt6 Play an Important Role in the Elongation of egr1 Transcription in Response to Serum Stimulation—Next we wanted to determine how SSRP1 and Spt6 regulate the expression of egr1 by performing a set of ChIP analyses. Fig. 5A shows the location of five amplicons used in real time PCR quantification of the ChIP-enriched DNA. Region 1 is a control region, which is far upstream of the egr1 gene. Regions 2 and 3 are in the egr1 promoter region and contain 4 and 2 CArG-boxes, respectively. Regions 4 and 5 are in the egr1 coding region. To catch the temporal distribution of the protein on the egr1 gene, we performed cross-linking and ChIP experiments at various times after serum stimulation. The corresponding transcription level of egr1 is shown in Fig. 5B. After 30 min of serum stimulation, the egr1 transcript was readily detected.

Both endogenous SSRP1 and Spt6 levels at the downstream coding regions of the egr1 gene (region 4 and particularly region 5) remarkably increased after serum stimulation compared with the nonstimulated control (0 min) (Fig. 5, C and D), suggesting that SSRP1 and Spt6 are recruited to the elongation complex after serum stimulation in a time-dependent fashion. The recruitment of SSRP1 and Spt6 to the egr1 coding region (region 5) is specific as they did not increase at a similar position of the c-myc gene, which is a late serum-responsive gene (41) (supplemental Fig. S2). Given the knowledge that SSRP1 is a co-activator for SRF (30) and that the egr1 promoter contains six SRF-binding sites (CArG-box) (38), it was surprising that there was no apparent recruitment of SSRP1 to the promoter region after serum stimulation (Fig. 5C). To determine the reason for this lack of recruitment, we also checked the occupation status of SRF on the egr1 gene before and after serum stimulation. Surprisingly, SRF was already bound to the egr1 promoter (regions 2 and 3) under nonserum stimulation conditions, and serum stimulation only slightly strengthened SRF binding to the egr1 promoter (Fig. 5E). Thus it is possible that this process does not require SSRP1 or that SSRP1 plays a transient function.

The carboxyl-terminal domain (CTD) of the largest subunit of RNA pol II is comprised of multiple heptad repeats (YSPTSPS motifs), and its phosphorylation has been shown to be mediated by TFIIH at transcriptional initiation (42, 43) and by CDK9 during transcriptional elongation (44). Two major phosphorylations of the CTD happen in vivo. Phosphorylation of the CTD on Ser-5 occurs at the promoter region and mediates recruitment and regulation of the mRNA capping enzyme guanylyltransferase; and phosphorylation of CTD on Ser-2 occurs on the elongating polymerase and couples transcription and 3′ end processing (45). To investigate further how SSRP1 and Spt6 are involved in the transcription elongation complex, we analyzed the effect of simultaneous depletion of SSRP1 and Spt6 on the recruitment of RNA pol II Ser-2 phospho-isofrom to the elongating region of the egr1 gene after serum stimulation. First, we checked the distribution of RNA pol II Ser-2 phospho-isofrom on the egr1 gene before and after serum stimulation. As shown in Fig. 5F, phosphorylation of RNA pol II on Ser-2 occurred on the promoter (region 3) and coding regions (region 4 and 5) after serum stimulation. In agreement with previous studies (34, 46), this RNA pol II Ser-2 phospho-isofrom was more abundant at the 3′ end of the gene (regions 4 and 5) than in the promoter region (region 3) after serum stimulation (Fig. 5F). When SSRP1 and Spt6 were depleted in the cells, much less RNA pol II Ser-2 phospho-isofrom was associated with the downstream coding region (region 5) (Fig. 6). These results suggest that the progression of RNA pol II to the downstream coding region was facilitated by SSRP1 and Spt6.
also showed a gene-specific requirement for FACT during transcription (47). The best example, as further examined here, is egr1, whose expression in response to serum stimulation requires both SSRP1 and Spt16, as knocking down either of these two proteins drastically inhibited the serum-responsive expression of egr1 (Fig. 4).

An intriguing question is how egr1 might be rapidly transcribed under conditions of serum stimulation. The high level of SRF on the egr1 promoter region before serum stimulation suggests the presence of a preassembled initiation complex (PIC). It has been shown that the transcription of human p21, c-fos, c-myc, and Drosophila hsp70 is regulated at post-initiation steps (48–50). Before stimulation, these promoters are preloaded with significant amounts of several components of the PIC, including RNA pol II itself. After stimulation, conversion of RNA pol II into a fully elongating form is achieved, which leads to rapid activation. Our data suggest that egr1 is probably regulated in the same fashion. Before serum stimulation, SRF exists on the egr1 promoter. The presence of SRF at the egr1 promoter before serum stimulation indicates that SRF may play an important role for the PIC assembly. After serum stimulation, rapid transcriptional activation is likely fulfilled by the conversion of RNA pol II to the elongation form. Although this model needs to be tested, our data suggest that the progression of this elongating RNA pol II on the egr1 coding region requires FACT (SSRP1 and Spt16) complex. FACT has been shown to facilitate transcriptional elongation by disassembling nucleosomes in vitro (10). Moreover, FACT has been shown to be involved in the regulation of other rapid inducible genes. In Drosophila, FACT is involved in the rapid induction of Hsp70 under thermal stress and displays kinetics of recruitment to the coding region of the hsp70 gene (25). Similarly in human cells, FACT is recruited to the coding region of egr1 after serum stimulation in a time-dependent fashion and contributes to the progression of RNA polymerase II along the egr1 gene. Hence, by participating in the regulation of the elongation of the egr1 transcription, FACT is essential for the expression of this immediate early serum-responsive gene, although it remains to be elucidated how FACT becomes committed to the regulation of a specific gene. It was suggested that histone H2B monoubiquitination facilitates FACT function (51, 52). So it is possible that after serum stimulation, the egr1 gene locus is one of the first under extensive histone tail modification and then recruits FACT to dissemble the nucleosome.

Another finding from our study was that a subset of the common target genes for SSRP1 and Spt16 was up-regulated when either SSRP1 or Spt16 was knocked down (Fig. 2, supplemental Fig. S1 and Tables 1–3). These data are consistent with the previous reports showing that the yeast orthologs of human SSRP1 and Spt16 were involved in down-regulation of specific genes (12) and that yeast Spt16 depletion causes YAT1 mRNA levels to increase (47). These results suggest that SSRP1 and Spt16 may act as co-repressors as well. It has yet to be determined if this repression is at the initiation or elongation step of transcription.

Interestingly, our study also suggests that SSRP1 and Spt16 may have independent functions in cells. Validating some of the SSRP1-specific target genes, such as plau, id2, or diusp5, further
Transcription Regulation by SSRP1 and Spt16

ACKNOWLEDGMENTS—We thank Mu-shui Dai for technique help in real time PCR, Robert Searles and Seeyan Lao for the microarray data analysis, and Jayme Gallegos for proofreading the manuscript.

REFERENCES

1. Formosa, T. (2003) Curr. Top Microbiol. Immunol. 274, 171–201
2. Singer, R. A., and Johnston, G. C. (2004) Biochim. Biophys. Acta 1682, 419–427
3. Vignali, M. H. A., Neely, K. E., and Workman, J. L. (2000) Mol. Cell. Biol. 20, 1899–1910
4. Svejstrup, J. Q. (2002) Curr. Opin. Genet. Dev. 12, 156–161
5. Akey, C. W., and Luger, K. (2003) Curr. Opin. Struct. Biol. 13, 6–14
6. Belotserkovskaya, R., and Reinberg, D. (2004) Curr. Opin. Genet. Dev. 14, 139–146
7. Orphanides, G., Wu, W. H., Lane, W. S., Hampsey, M., and Reinberg, D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2307–2311
8. Orphanides, G., LeRoy, G., Chang, C. H., Luse, D. S., and Reinberg, D. (1998) Mol. Cell. Biol. 18, 105–116
9. Bruhn, S. L., Pil, P. M., Essigmann, J. M., Housman, D. E., and Lippard, S. J. (1999) Nature 400, 284–288
10. Belotserkovskaya, R., Oh, S., Bondarenko, V. A., Orphanides, G., Studitsky, V. M., and Reinberg, D. (2003) Science 301, 1090–1093
11. Malone, E. A., Clark, C. D., Chiang, A., and Winston, F. (1991) Mol. Cell. Biol. 11, 5710–5717
12. Chamber, C. D., and Johnson, G. C. (1991) Mol. Cell. Biol. 11, 5718–5726
13. Evans, J., Brewster, N. K., Xu, Q., Rowley, A., Altheim, B. A., Johnston, G. C., and Singer, R. A. (1998) Genetics 150, 1393–1405
14. Chait, J., and Tarnawski, A. S. (2002) J. Biol. Chem. 277, 22793–22799
15. Formosa, T., Ruone, S., Adams, M. D., Olsen, A. E., Eriksson, P., Yu, Y., and Stillman, D. J. (2005) Mol. Cell. Biol. 25, 5812–5822