MOLECULAR AND CELLULAR BIOLOGY, May 2004, p. 3734–3746 Vol. 24, No. 9
0270-7306/04/$08.00+0 DOI: 10.1128/MCB.24.9.3734–3746.2004

Suppressor of sable, a Putative RNA-Processing Protein, Functions at the Level of Transcription

Yung-Shu Kuan,† Paul Brewer-Jensen, and Lillie L. Searles*

Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-3280

Received 28 August 2003/Returned for modification 13 October 2003/Accepted 30 January 2004

The *Drosophila melanogaster su(s)* gene product negatively regulates the expression of mutant alleles with transposon insertions in the 5′-transcribed region by an unknown mechanism. We have investigated here *su(s)* function through in vivo structure-function analysis, heterologous reporter gene assays, and in vivo transcriptional induction experiments. We have shown that mutations of two arginine-rich motifs (ARMs), an acidic region, or two CCCH zinc fingers affect the ability of Su(s) to downregulate the expression of an insertion mutant allele and to autoregulate genomic *su(s)* transgenes. Using yeast and HeLa cell assays, we found that, when tethered to the promoter region, the N- and C-terminal regions of Su(s) can repress reporter gene expression, and all three motifs, but most significantly the ARMs, contribute to the repression activity. Finally, we showed that, in vivo, Su(s) inhibits the transcriptional induction of a transgene with an insertion in the first exon but does not affect induction of a similar transgene with a consensus 5′ splice site near the upstream boundary of the insertion. Together, these results reveal a link between Su(s), transcription, and pre-mRNA processing.

In eukaryotes, control mechanisms operate at various stages of gene expression to generate specific and dynamic patterns of protein accumulation. Efficient mRNA production depends on complex interactions between a large number of components that regulate pre-mRNA synthesis and processing in time and space. The current understanding of eukaryotic gene expression regulation has been derived primarily from experiments performed in relatively simple systems, e.g., in vitro, cultured cell lines or single-cell eukaryotes. Although much progress has been made and important insights have come from these studies, the view of how various mRNA metabolic pathways are coordinated and integrated under normal physiological conditions and during development is incomplete. The analysis of genetic regulatory processes in model eukaryotic organisms such as *Drosophila melanogaster* can contribute to the understanding of more complex aspects of regulation that cannot be studied in simpler model systems.

Several pre-mRNA transcription and processing regulators in *D. melanogaster* have been identified by virtue of the fact that mutations in genes encoding these proteins can suppress or enhance the effects of transposon-induced mutations. One such gene is *su(s)*. It encodes a 150-kDa nuclear protein that binds to RNA in vitro (28, 39). Loss-of-function *su(s)* mutations alter the phenotypes of several mutant alleles of other genes that are associated with transposon insertions (37, 38). Genetic studies have shown that *su(s)* mutations enhance the mutant phenotypes of alleles of *cut* (ct^k), *forked* (f^1), and *bithorax* (bx^1, bx^3, and bx^{34e}) but suppress the mutant phenotypes of alleles of *sable* (s^1), *vermilion* (v^1, v^2, and v^k), *yellow* (y^{76d28}), and *purple* (pr^{bw}) (17, 20, 24, 35, 36). In an otherwise wild-type background, a homozygous null *su(s)* mutant exhibits reduced viability, and males are sterile when reared at low temperatures (42). Ectopic overexpression of Su(s) is lethal (38).

Molecular studies have shown that Su(s) negatively regulates the amount of RNA generated by mutant alleles that have transposon insertions located in the 5′-transcribed region. The transposon insertion in each allele is positioned in the opposite transcriptional orientation as the affected gene. The *su(s)*-suppressible alleles v^k, y^{76d28}, and pr^{bw} produce higher steady-state levels of mRNA in a *su(s)* mutant background, i.e., when Su(s) function is impaired, than in a $su(s)^+$ background (14, 17, 20). The v^k allele normally produces a barely detectable level of RNA that is nearly wild-type in length, the majority of transposon sequences having been removed by splicing at cryptic splice sites near the transposon ends. The level of v^k RNA accumulation is lower in $su(s)^+$ than *su(s)* mutant flies. However, a v^k derivative with a consensus (instead of a cryptic) 5′ splice site at the upstream boundary of the insertion produces the same, high level of *v* RNA in the presence or absence of *su(s)* product (15). These results suggest that the efficiency of splicing complex assembly in the 5′ region can influence Su(s)-mediated regulation of v^k RNA levels.

Since modulation of RNA levels by Su(s) depends on transcribed sequences, our lab and others concluded that Su(s) most likely influences RNA stability. However, two recent insights have prompted us to reconsider the possibility that Su(s) negatively regulates transcription of the insertion mutant alleles. First, a substantial body of recent data indicates that transcription and pre-mRNA processing are intimately coupled in vivo (7, 33). During the elongation phase of transcription, RNA processing components associate with the phosphorylated C-terminal domain (CTD) of the largest subunit of RNA polymerase II (RNAP II). These interactions facilitate the assembly of processing components onto the pre-mRNA as it is being synthesized, and it appears that capping, polyadenylation, and at least the initial stages of splicing complex

* Corresponding author. Mailing address: Department of Biology, CB#3280, UNC at Chapel Hill, Chapel Hill, NC 27599-3280. Phone: (919) 966-4989. Fax: (919) 962-1625. E-mail: lsearles@email.unc.edu.
† Present address: Department of Embryology, Carnegie Institution, Baltimore, Md.

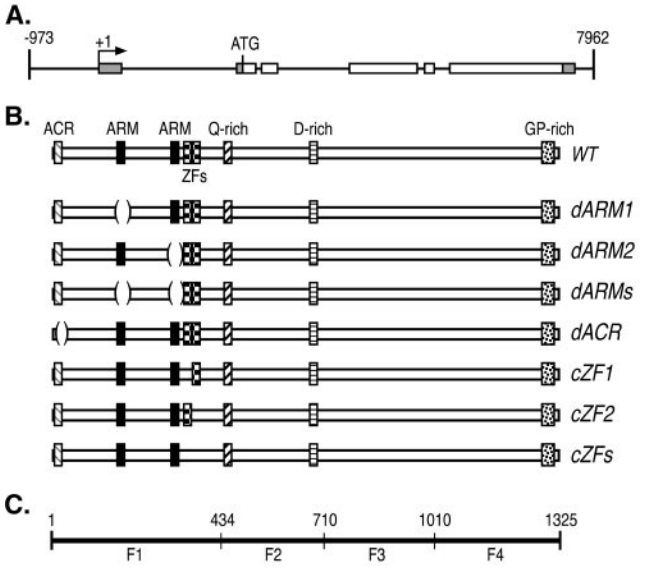

FIG. 1. Schematic drawings of a *su(s)* genomic clone and Su(s) protein. (A) The 8.9-kb genomic clone used for creating transgenic fly lines. Transcription starts at +1. Exons are shown as rectangular boxes. Shaded boxes indicate the 5′- and 3′-untranslated regions and open boxes indicate the protein coding region. Introns are shown as thin lines. (B) Full-length Su(s), a 1,325-aa protein, contains the following structural features: an ACR, two ARMs, two ZFs, a glutamine-rich region (Q-rich), an aspartic acid-rich region (D-rich), and a glycine/proline-rich region (GP-rich). F1 to F4 are fragments used in the reporter gene assays (see Fig. 5).

assembly occur during transcription. Second, our lab has shown that Su(s) associates with polytene chromosomes (28, 38) and colocalizes with a form of RNAP II that has hypophosphorylated CTD repeats (4; unpublished observations). RNAP II is believed to be hypophosphorylated at initiation and during early elongation phases of transcription. Thus, it is possible that Su(s) functions during transcription by a mechanism that, at least in some instances, is connected to splicing complex assembly on the nascent transcript. Therefore, analysis of Su(s) may reveal new insights into the transcription-RNA processing coupling mechanisms.

Su(s) has limited similarity with proteins outside of insects. This 1,325-amino-acid (aa) protein contains several regions of low sequence complexity, as well as short regions that encode known functional motifs (Fig. 1B). An acidic region (ACR) is found at the amino terminus (aa 12 to 29). Acidic domains are found in many transcription factors, including VP16 (8), E2F1 (21), and the largest subunit of RNAP II (40). Two arginine-rich motifs (ARMs; aa 151 to 168 and aa 269 to 294), also located in the amino-terminal portion of Su(s), are similar to motifs found in RNA-binding proteins, such as human immunodeficiency virus Tat (10) and Rev (23). Two tandem $CX_8CX_5CX_3H$ zinc fingers (ZFs; aa 336 to 378) are located just downstream of the ARMs. Multiple copies of this type of ZF are found in the *Caenorhabditis elegans* transcriptional repressor PIE-1 (27), the splicing factor U2AF 35-kDa subunit (36), the polyadenylation factor CPSF 30-kDa subunit (5), and the mammalian RNA stability factor TTP (10, 23). Previously, our lab demonstrated that the two ARMs mediate the in vitro RNA binding of Su(s) (28, 38). However, a function has not been established for these other putative motifs.

In previous Su(s) structure-function studies, wild-type and ARM deletion mutant *su(s)* cDNA transgenes were ectopically expressed by using the two-part Gal4/UAS system (38). None of these combinations expressed *su(s)* in its normal pattern and, most significantly, this analysis was complicated by the fact that certain Gal4 driver/*UAS-su(s)* combinations were lethal. Furthermore, none of the Gal4 drivers directed *su(s)* expression in the pattern needed to restore normal regulation of the v^k allele. These complications made it difficult to evaluate the biological consequences of the ARM deletions in vivo.

We have used here the endogenous *su(s)* promoter and regulatory sequences to drive expression of *su(s)* genomic transgenes. This has allowed us to determine the effect of mutating the motifs mentioned above on Su(s) function. This analysis revealed that Su(s) autoregulates the level of its own transcript. Furthermore, we have shown that the ARMs, ACR, and ZFs contribute to Su(s)-mediated downregulation of both of *su(s)* and v^k RNAs. In a second line of experiments, we used yeast and HeLa cell reporter gene expression assays to test the hypothesis that Su(s) modulates transcription. We found that N- and C-terminal fragments of Su(s), when tethered to the promoter region, each repress reporter gene expression. All three motifs were implicated in repression in these assays. However, the contribution of the ARMs to repression was most significant. Lastly, we examined how the presence or absence of Su(s) affects the transcriptional induction of v^k transgenes that have transposon insertions in the 5′-transcribed region. These experiments indicate that Su(s) negatively regulates the induction of a transgene with cryptic 5′ splice sites at the upstream boundary of the inefficiently spliced "transposon intron." In contrast, Su(s) does not interfere with the induction of a similar transgene with a consensus 5′ splice site in the place of one of the cryptic 5′ splice sites. Taken together, these results provide strong evidence that Su(s) regulates transcription or a process that is mechanistically linked to transcription. Furthermore, it appears that Su(s) acts at a stage in gene expression where transcription and pre-mRNA processing pathways converge.

MATERIALS AND METHODS

DNA construct generation. DNA fragments with mutations in *su(s)* were generated by using the overlapping PCR technique (19). Subsequently, each mutated fragment was substituted into a wild-type clone by using convenient restriction sites. The ARM1 deletion removes Su(s) aa 151 to 168, the ARM2 deletion removes aa 269 to 294, and the ARM deletion removes both of these regions. The ZF1 mutant construct has a missense mutation at aa 350 (Cys to Gly), the ZF2 mutant has mutations at aa 374 (Cys to Gly) and aa 378 (His to Gly), and the ZF mutant has all three changes. The ACR deletion removes aa 12 to 29. The wild-type and mutant *su(s)* transgenes were made by inserting the 8.9-kb *su(s)* genomic DNA fragment, which includes sequences from the −973 nucleotide to the +7962 nucleotide into the germ line transformation vector pCaSperXN (R A. Voelker, unpublished data). The clones used for the yeast reporter gene expression assays were constructed as follows. The wild-type *su(s)* cDNA fragment encoding aa 1 to 434 (F1) was obtained from pMAL-SU(S)$_{1-434}$ (38). Fragments encoding aa 434 to 710 (F2) and aa 1010 to 1325 (F4) were obtained from the cDNA clone p15-1 (Voelker, unpublished). The coding region for aa 710 to 1010 (F3) was obtained from p62-11 (37). These fragments were cloned into the pGBT9 vector (Y. Xiong, unpublished data) by standard molecular biology techniques. Downstream Gal4 activation domain (Gal4AD) fusions were generated by PCR with primer sets designed to create in-frame cloning sites. Clones for reporter gene expression assay in HeLa cells were generated by

utilizing the strategy described above. The vector Gal4BD plasmid containing Gal4 aa 1 to 95 and reporter pGAL4-TKCAT and pBLCAT plasmids were provided by T. K. Blackwell (6). Plasmid pDMNT3L1-195 was a gift from P. Peterson (1). All mutations and clones were verified by DNA sequencing.

Generating fly stocks and brown eye-pigment quantitation. P-element mediated germ line transformations were performed basically as described by Rubin and Spradling (34). Crosses with standard balancer chromosomes were used to generate homozygous transgenic lines in the *yw* [*su(s)*$^+$] background. The crosses for generating *su(s)*$^-$ *v*^{k}/[*su(s) w*$^+$] flies were as follows. The null allele *su(s)*^{R39} was apparently lethal in the TM3 balancer background. Therefore, we generated the fly line *su(s)*^{R39} *v*^{k}/FM7; +/D for subsequent crosses with flies bearing transgenes on the third chromosome. First, *su(s)*^{R39} *v*^{k}/FM7; +/D female flies were crossed to *yw*; [*su(s) w*$^+$]/[*su(s) w*$^+$] male flies to generate *yw*/FM7; [*su(s) w*$^+$]/D female flies and *su(s)*^{R39} *v*^{k}; [*su(s) w*$^+$]/D male flies, which were then crossed to each other to generate *su(s)*^{R39} *v*^{k}/FM7; [*su(s) w*$^+$]/[*su(s) w*$^+$] female flies. This was followed by crossing to *su(s)*^{R39} *v*^{k}; [*su(s) w*$^+$]/D male flies to generate *su(s)*^{R39} *v*^{k}; [*su(s) w*$^+$]/P-[*su(s) w*$^+$] male and female flies. The transgenes on the second chromosome were introduced into *su(s)*^{R39} *v*^{k} background utilizing the same strategy. All fly lines were further confirmed by PCR or DNA sequence analysis. Brown eye pigment quantitation was conducted as previously described in Protocol 132 by Ashburner (2).

Protein level analysis. For each transformant line examined, 20 adult flies, 1 to 2 days old, were collected and ground in 200 μl of 1.5× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer without bromophenol blue, boiled for 10 min, and microcentrifuged at 15,000 × *g* for 10 min to pellet cellular debris. Protein concentration was determined by using the Bradford assay (Bio-Rad), and 35 μg of protein was subjected to SDS-PAGE and then blotted onto polyvinylidene difluoride (Hybond-P; Amersham). The blot was probed with a 1:1,000 dilution of polyclonal antibodies raised against aa 42 to 146 of Su(s). A horseradish peroxidase-conjugated anti-rabbit secondary antibody (Amersham) was used at a 1:2,000 dilution, and signals were detected by enhanced chemiluminescence (Amersham). The level of Gal4-Su(s) fusion protein was evaluated in HeLa cells as described by Batchelder et al. (6). Blots were probed with a 1:250 dilution of polyclonal antibodies raised to aa 1 to 147 of Gal4 (Santa Cruz) and detected as described above.

RNA analysis. For analysis of *v* and *su(s)* expression, total RNA preparations were performed as described by D'Avino et al. (12) with 10 flies for each line examined. For the reverse transcription-PCR (RT-PCR) assay, 5 μg of total RNA was used for each line, and the reaction was performed as described by Burnette et al. (9). Under our conditions for RNA isolation and RT, PCR amplification of wild-type *v* and *su(s)* transcripts was exponential through the 26th cycle (data not shown). For all of the experimental determinations, the quantitative comparison between fly lines was performed on samples amplified through the 23rd cycle, which was within the linear range in every case. For Northern blot analysis, ca. 6 μg of total RNA was utilized for each line, and the experiments were performed as described by Fridell and Searles (15). All quantitation results were determined from three independent experiments utilizing a Storm 800 PhosphorImager with ImageQuant5.2 analysis software (Molecular Dynamics). Primers used for amplifying *v* transcripts were V575F (5′-AGG AAA CGG AAA CGA TCA CGA TGA-3′) and V1095R (5′-CCG AGT TGC GAA TCG AAT TCC GCG CCT-3′). This set generates a 520-bp cDNA fragment, which is 188 bp shorter than the corresponding fragment generated by amplification of *v* genomic DNA. The primers used for amplifying *su(s)* transcripts were 3062F (5′-CCC TTA ATC AGC AAT CTT CAA AAA TCC AAG-3′) and 4329L (5′-CGT TCA TCA TCT CAT ATT CG-3′). This set generates a 502-bp cDNA fragment, which is 765 bp shorter than the corresponding genomic DNA *su(s)* fragment. The primers used for amplifying the *rp49* transcript were RP49F (5′-ATC CGC CCA GCA TAC AGG-3′) and RP49R (5′-CTC GTT CTC TTG AGA ACG CAG-3′). This set generates a 396-bp cDNA fragment, which is 63 bp shorter than the fragment generated from *rp49* genomic DNA.

Analysis of *Mtn v*^{k} *LTR* and *Mtn v*^{k} *LTR 5′con* expression was performed on RNA isolated from 0- to 4-day-old adult flies. To examine the effect of induction with various amounts of copper, flies were reared on standard food and then transferred to media prepared by combining equal volumes of the instant *Drosophila* medium (Formula 4-24; Carolina Biological Supply) and a solution of 0 to 0.8 mM $CuSO_4$ for 24 h. In the induction time course experiments, flies were placed in empty bottles for 2 h prior to being placed on medium containing 0.5 mM $CuSO_4$. Flies were collected at the specified times and stored at −70°C. The procedures used to generate the transgenic constructs, the total RNA isolation procedure, and the Northern blotting procedure were described previously (14, 15). Polyadenylated mRNA was purified by using the PolyATract mRNA Isolation System IV (Promega).

TABLE 1. Summary of the *su(s)* transformant lines used in this study[a]

Transformant line	Chromosomal position	Insertion copy no.
WT#1	3rd	1
WT#2*	2nd	1
dARM1#1	2nd	2
dARM1#2	3rd	1
dARM2#1*	3rd	1
dARM2#2	3rd	2
dARMs#1	2nd	2
dARMs#2	2nd	1
dACR#1	2nd	2
dACR#2*	3rd	1
cZF1#1	2nd	1
cZF1#2	2nd	1
cZF2#1	3rd	2
cZF2#2*	2nd	2
cZFs#1*	3rd	1
cZFs#2*	2nd	1

[a] The chromosomal position of each transgenic insertion was determined by using standard crosses to balancer stocks. The insertion copy number was determined by Southern blot analysis. *, The transformant line exhibits poor viability in the *su(s)*^{R39} (null mutant) background.

Polytene chromosome staining. Salivary glands were dissected from third-instar larvae and immunostained as described previously (4) except that 3% normal donkey serum in PBT (0.1% Triton X-100 in phosphate-buffered saline) was used as a nonspecific blocker before and during exposure to antisera. Antisera dilutions used for chromosome staining were 1:150 for polyclonal rabbit anti-*su(s)*; 1:50 for polyclonal goat anti-RNAP IIA; 1:200 for donkey anti-rabbit and donkey anti-goat secondary antibodies conjugated with either Cy3 or Cy2 fluorescein (Jackson ImmunoResearch Laboratories, Inc.). Colocalization was determined by the color of the signal in merged images. The appearance of yellow indicates that the red and green signals are roughly equivalent. Multiple nuclei of each genotype were examined in at least three independent experiments. The anti-RNAP IIA polyclonal antibody was raised against unphosphorylated CTD repeats but may also recognize RNAP II with partially phosphorylated repeats.

Yeast cell culture and β-galactosidase assays. Yeast (PJ69-4a) cells, grown in yeast extract-peptone-dextrose medium, were transfected by the polyethylene glycol-lithium acetate precipitation method (Yeast Handling Book; Clontech) with 0.1 μg of effector plasmid and 100 μg of carrier DNA (Herring Testes DNA; Clontech), followed by growth on the appropriate selective plates. Positive colonies were picked and respread onto new selective plates. β-Galactosidase activity measurements were performed according to procedures described elsewhere (3).

HeLa cell culture and CAT assays. HeLa cell culture and transfection were performed essentially as described by Batchelder et al. (6). For the HDAC dependency assay, the HDAC inhibitor Trichostatin A (Biomol Research Laboratories Inc) was added 24 h posttransfection to a final concentration of 100 nM. Cells were harvested and chloramphenicol acetyltransferase (CAT) assays were performed on 40 μg of protein (measured by the Bradford assay) by using a liquid scintillation assay technique described by the manual from Promega. β-Galactosidase reporter plasmids (pCMV-βGal; Applied Biosystems) were cotransfected to provide an internal reference for transfection efficiency. All transfection experiments were performed at least three times in duplicate.

RESULTS

Mutations of the ARMs, ACR, and ZFs affect Su(s)-mediated repression of *v*^{k} expression. For Su(s) structure-function analysis, we made several genomic *su(s)* constructs with alterations affecting the coding region and transformed these into the *Drosophila* germ line (34) (Fig. 1B) (Table 1). These transgenes were designed to produce Su(s) with a deletion of either one or both ARMs (dARM1, dARM2, and dARMs, respectively), with a deletion of the ACR (dACR), or with point

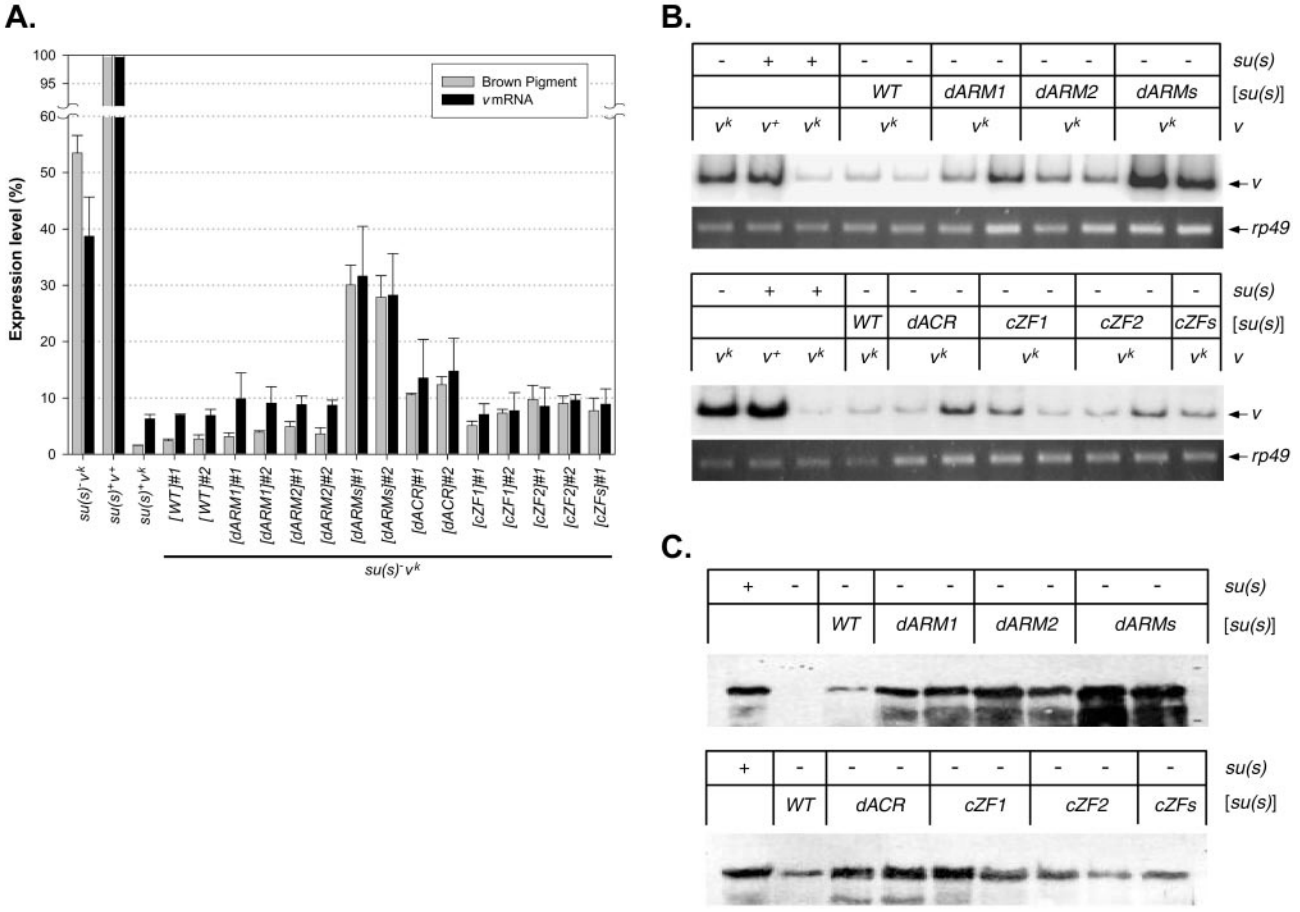

FIG. 2. The dACR and dARMs mutant Su(s) proteins exhibit defects in repressing v^k mRNA accumulation. All *su(s)* transgenes were evaluated in the $su(s)^- v^k$ background. (A) Brown eye pigment and *v* mRNA levels of *su(s)* transformant lines were compared to the $su(s)^+ v^+$ (Oregon R) control, which was set as 100%. Each datum point represents the mean from three independent experiments. (B) Representative RT-PCR experiments. Radioactively labeled *v* RT-PCR products (top panel of each set) were subjected to electrophoresis on an 8% polyacrylamide gel, and the image was visualized and quantitated by using a PhosphorImager. RT-PCR products for the internal control *rp49* (bottom panel of each set) were applied to a 1.2% agarose gel, and ethidium bromide-stained DNA was visualized and quantitated by using the Eagle Eye Still Video System. The genetic backgrounds of the RNA samples used for the RT-PCRs are indicated above the lanes as follows: *su(s)*, the endogenous gene; and [*su(s)*], the transgenes. (C) Total protein preparations were separated by SDS–8% PAGE gel and blotted onto a polyvinylidene difluoride membrane. Su(s) protein was detected by probing with polyclonal antibodies raised against Su(s) aa 42 to 146 and visualized by enhanced chemiluminescence. In panels B and C, when two lanes are shown for a given transgene, each lane represents a different transformant line.

mutations in critical cysteine residues of either one or both ZFs (cZF1, cZF2, and cZFs, respectively).

The initial assessment of Su(s) function involved evaluating the capability of each *su(s)* transgene to modulate expression of the v^k mutant allele, which has a full-length *412* retrotransposon inserted into the first *v* exon (14). The *v* gene encodes tryptophan oxygenase, an enzyme that is required for the brown eye-pigment synthesis (36). Su(s) normally represses expression of the v^k allele. Thus, the amount of brown eye pigment in v^k mutant flies is inversely proportional to the level of Su(s) activity. To evaluate the function of Su(s) derivatives with mutations in putative functional domains, we performed brown pigment assays (2) on v^k mutant fly stocks that were homozygous for an autosomal *su(s)* transgene (indicated by brackets), with a null *su(s)* allele at the endogenous *su(s)* locus on the *X* chromosome. The results of this analysis are shown in Fig. 2A. In these experiments, the brown eye-pigment level in control $su(s)^+ v^k$ flies [1.5% of the $su(s)^+ v^+$ level] was used as the reference point for comparison of the transgenic lines.

A wild-type *su(s)* transgene (*[WT]*) restored repression of v^k expression, resulting in production of a very low amount of pigment, i.e., only about twice the $su(s)^+ v^k$ level. This indicates that the wild-type transgene contains the *su(s)* regulatory elements needed to restore Su(s) expression and activity to nearly normal levels. In $su(s)^- v^k$ flies, which completely lack Su(s), brown pigment levels were elevated 36-fold. None of the mutant transgenes were as deficient in repression activity as the *su(s)*-null allele. However, v^k flies expressing Su(s) lacking both ARMs (*[dARMs]*) produced 19 times as much brown pigment as $su(s)^+ v^k$ flies. In contrast, the brown pigment levels of v^k mutants carrying transgenes with deletions in only one ARM (*[dARM1]* or *[dARM2]*) were not significantly different from *[WT]*. Thus, neither deletion alone substantially impairs Su(s) function. Deleting the acidic region (*[dACR]*) produced

an eightfold increase in pigment levels ($P = 2.3E-06$), whereas the single and double ZF mutations (*[cZF1]*, *[cZF2]*, and *[cZFs]*) caused modest four- to sixfold increases in pigment levels ($P \leq 0.004$). This suggests that these domains play a role in the activity of Su(s) but that substantial residual function remains in their absence. Western blot analysis showed that the Su(s) protein level in each transformant line was either similar to or higher than the protein produced in wild-type flies (Fig. 2C). Therefore, the failure to rescue the $su(s)^- v^k$ phenotype is not due to lower levels of protein made from the transgenes.

To examine the effect of Su(s) on the accumulation of v^k mRNA, we used RT-PCR to quantitate v^k mRNA levels in total RNA samples isolated from these same transformant lines (Fig. 2A and B). As was done in the analysis of pigment levels, we compared the transgenic *[su(s)]* v^k RNA levels to the $su(s)^+ v^k$ level (the latter being 6.3% of the $su(s)^+ v^+$ level). We found that flies carrying a wild-type *su(s)* transgene accumulated the same low level of v^k mRNA as $su(s)^+ v^k$ flies. The $su(s)^- v^k$ flies, which completely lack Su(s) activity, showed a 6.2-fold increase in v^k mRNA. The levels of v^k mRNA in *[dARM1]* or *[dARM2]* flies were not significantly different from the $su(s)^+ v^k$ level. However, the double deletion mutant *[dARMs]* flies showed a 4.7-fold increase of v^k mRNA. The v^k mRNA level in *[dACR]* flies was elevated twofold ($P = 0.02$). The v^k mRNA levels in flies carrying the single or double ZF mutant transgenes were not significantly different from the $su(s)^+ v^k$ control. Thus, the results of the eye pigment and *v* mRNA assays were qualitatively similar. However, the pigment assay, whereas a more indirect measure of Su(s) activity, was apparently better for detecting and quantifying subtle differences at the lower range of v^k expression. Taken together, both sets of experiments indicate that the ARMs, ACR, and ZFs, in descending order of significance, contribute to Su(s)'s activity in repressing expression of the mutant v^k allele.

Mutations of the ARMs and ZFs have a more substantial impact than the ACR on the autoregulatory activity of Su(s). Previously, Turnage et al. (38) showed that Su(s), when ectopically expressed using the Gal4/UAS system, downregulates the production of RNA from *UAS-su(s)* transgenes and that the ARMs are important for this inhibitory effect. Since the transgenic *su(s)* constructs used in this earlier study were cDNA clones expressed under the control of a heterologous promoter, it was unclear whether Su(s) regulates production of its own RNA under normal conditions. In the course of the structure-function analysis described above, we noticed that the level of Su(s) protein produced by some of the mutant transgenes was higher than normal. This was most apparent for the ARM double deletion mutant [*dARMs]* flies (see Fig. 2C). We reasoned that if Su(s) negatively regulates the synthesis or stability of its own RNA, then flies producing only mutant Su(s) should exhibit higher levels of *su(s)* RNA per *su(s)* gene copy than flies producing normal Su(s). Thus, we compared the *su(s)* mRNA level per gene copy in total RNA isolated from homozygous transgenic lines in both $su(s)^+$ and $su(s)^-$ backgrounds. The mRNA level per gene copy from $su(s)^+$ flies (two copies) was used as the reference point. Northern blot analysis (Fig. 3) showed that *WT* mRNA accumulated at essentially the same level per gene as $su(s)^+$ RNA in either $su(s)^+$ or $su(s)^-$ backgrounds. Furthermore, flies carrying both the wild-type

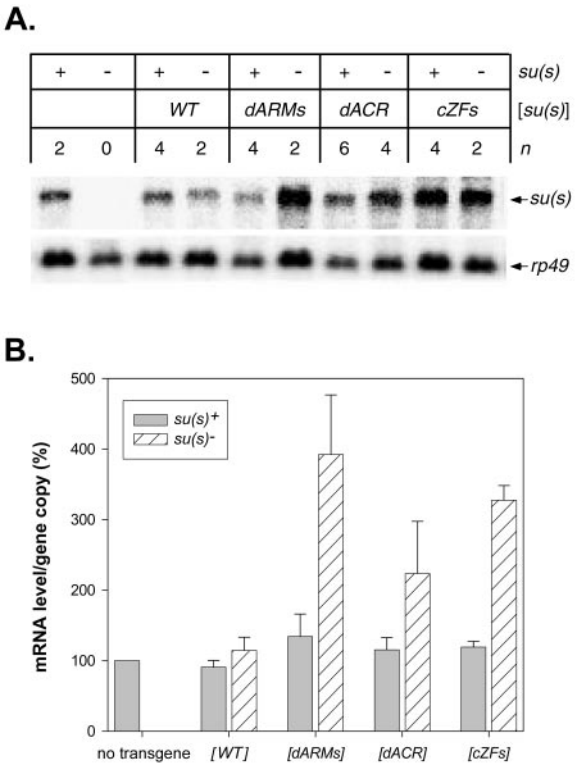

FIG. 3. Su(s) regulates its own mRNA production. (A) A representative Northern blot experiment. Samples of total adult RNA were separated in a 1.5% formaldehyde agarose gel and blotted onto nylon membrane. *su(s)* and *rp49* mRNAs were detected with ^{32}P-labeled antisense RNA probes, and signals were visualized and quantitated as described in Fig. 2. *su(s)* is the endogenous gene, *n* represents the *su(s)* gene copy number, and *[su(s)]* represents the transgenes. (B) The amount of *su(s)* mRNA per gene copy in each transformant line was compared to the amount per gene copy in the $su(s)^+$ line, which was set as 100%. The internal control *rp49* was used to control for loading differences between samples. Each datum point represents the mean from three independent experiments.

allele of endogenous *su(s)* and any of the mutant transgenes produced the same level of *su(s)* mRNA per gene as $su(s)^+$ flies. This result is expected if Su(s) acts as a negative regulator and the activity of wild-type Su(s) is dominant to that of the mutant proteins. Also consistent with this hypothesis, *[dARMs]* and *[cZFs]* flies accumulated a threefold-higher level ($P \geq 0.004$) of *su(s)* mRNA in the $su(s)^-$ than in the $su(s)^+$ background. The *su(s)* RNA level in *[dACR]* flies was twofold higher ($P = 0.035$) in the $su(s)^-$ than in the $su(s)^+$ background. RT-PCR analysis produced similar results (data not shown). Together, these results provide additional evidence that Su(s) negatively regulates the accumulation of its own RNA. Furthermore, these data indicate that the ARMs and ZFs are more important for this autoregulatory process than the ACR.

The ARMs and ACR are required for the stable association of Su(s) with polytene chromosomes. Our lab previously used immunocytochemical analysis to demonstrate that Su(s) associates with a subset of polytene chromosome sites (28, 38). Anti-Su(s) antibodies produce a strong signal at a small number of sites and a weaker signal at other sites, and the strong Su(s) sites correspond to strong hypophosphorylated RNAP II

(RNAP IIA) sites (4; W. L. Bai et al., unpublished data). To determine whether particular domains of Su(s) are required for the association of Su(s) with polytene chromosomes or for RNAP IIA colocalization, we performed indirect immunofluorescence staining on polytene chromosomes obtained from one line for each *su(s)* transgene. We found that neither the dARM1 or dARM2 mutations (data not shown) nor the cZF1, cZF2 (data not shown), or cZFs double mutation (Fig. 4J) substantially affected the protein's ability to associate with chromosomes. In each case, the mutant Su(s) proteins colocalized with RNAP IIA (e.g., see Fig. 4J to L). However, dARMs, the double deletion derivative, and dACR both showed reduced binding to chromosomes (Fig. 4D and G). By staining whole salivary glands with anti-Su(s) antibody, we determined that the dARMs and dACR mutant proteins accumulate, as expected, in salivary nuclei (data not shown). This eliminates the possibility that the disrupted chromosome association is due to the exclusion of the proteins from the nucleus. Thus, the ARMs and ACR regions, which our expression studies defined as being involved in the repression activity of Su(s), are also required for the stable association of this protein with polytene chromosomes. The apparently normal binding activity of the cZFs mutated protein to polytene chromosomes suggests that these motifs play a distinctive role in the activity of Su(s), perhaps subsequent to its localization to chromosomes.

The N- and C-terminal regions of Su(s) strongly repress reporter gene transcription in both yeast and HeLa cells. It has been well established from previous studies and the results presented above that Su(s) negatively regulates RNA levels. However, it is not known whether this protein acts during transcription or posttrancriptionally, i.e., affecting RNA stability. Thus, we used two reporter gene expression assays to test the hypothesis that specific domains of Su(s) are capable of repressing transcription. One of these was the yeast one-hybrid assay (Fig. 5A). For this assay, we inserted fragments of the *su(s)* coding region (Fig. 1C) into an expression vector containing sequences encoding Gal4 DNA-binding and transcriptional activation domains (Gal4BD and Gal4AD, respectively). We transformed these *Gal4-su(s)* plasmids into yeast and measured their ability to activate a Gal4-dependent reporter gene (*Gal7-LacZ*). For the HeLa cell expression assay (Fig. 5B), these same *su(s)* fragments were cloned into an expression vector containing only the Gal4BD coding region. These constructs were transiently transfected into HeLa cells, along with a reporter gene with multiple Gal4 DNA-binding sites upstream of the thymidine kinase promoter, which contains both basal and enhancer elements (*GAL4-TKCAT*). Expression of *GAL4-TKCAT* is Gal4 independent but can be influenced by the binding of Gal4BD-fusion proteins carrying repression or activation domains. Control *Gal4* expression vectors lacking *su(s)* sequences and a reporter plasmid lacking Gal4 DNA-binding sites (HeLa assay only) were included in these experiments.

The results of this analysis are shown in Fig. 5C. We found that the N-terminal quarter of Su(s) (aa 1 to 434, F1) repressed transcription of the reporter gene 13-fold in yeast and 28-fold in HeLa cells. Likewise, the C-terminal quarter of Su(s) (aa 1010 to 1325, F4) repressed reporter gene transcription 30-fold in yeast and 50-fold in HeLa cells. F1 and F4 did not significantly repress transcription of a reporter gene without upstream GAL4 binding sites (BLCAT2; Fig. 5B) in HeLa cells. Thus, repression only occurred when these two Gal4BD-Su(s) fusions were tethered to the promoter region. Su(s) aa 434 to 710 (F2), when fusion to Gal4BD alone, self-activated reporter gene expression in yeast (data not shown), and an aspartic acid (D)-rich region (aa 643 to 662 [Fig. 1B]) was shown to mediate this effect. Therefore, we did not evaluate the repression activity of F2 in this system. In HeLa cells, F2 did not significantly influence CAT reporter gene expression. Su(s) aa 710 to 1010 (F3) did not affect reporter gene transcription in the yeast assay but enhanced the reporter gene transcription in HeLa cells approximately eightfold. This fragment also enhanced transcription of the reporter gene lacking Gal4-binding sites (BLCAT2) twofold in HeLa cells. Full-length Su(s) produced a modest (twofold) repressive effect on the reporter gene expression in HeLa cells. Western blotting (Fig. 5F) showed that most of the Su(s) fragments were expressed at similar levels. However, full-length Su(s) was expressed at a barely detectable level in HeLa cells (not shown); thus, the actual repression activity of the full-length protein in these cells may be greater than observed in these experiments. Together, these results indicate that the N- and C-terminal regions of Su(s) are capable of repressing gene expression at the level of transcription in both yeast and HeLa cells. Amino acids in the central portion of Su(s) are capable of activating or enhancing reporter gene expression in one assay or the other but not both.

The repression activities of Su(s) are largely HDAC independent. Some eukaryotic proteins repress transcription by recruiting histone deacetylase (HDAC) corepressors to a gene to modify chromatin structure (18, 29). Previous studies have shown that the incubation of HeLa cells in the HDAC inhibitor trichostatin A (TSA) can relieve HDAC-dependent repression of a CAT reporter gene (1). Thus, we used TSA to test whether the repression activities of F1 and F4 in HeLa cells are HDAC dependent (Fig. 5D). We found that both F1 and F4 showed strong repression (18- and 10-fold, respectively) in the presence of TSA, although the repression activity of F4 was reduced ~3-fold under these conditions. The repression activity of DNMT3L195, a mammalian protein that is known to recruit HDAC, was reduced 5.2-fold in the presence of TSA. TSA produced a 1.5-fold increase in reporter gene transcription in the absence of a repressor (GAL4BD). The results obtained with these positive and negative control samples are consistent with data from other labs (1, 13). Thus, these data indicate that the repression activity of F1 in HeLa cells is HDAC independent. Although a component of F4-mediated repression may be HDAC dependent, the majority of the repression activity of F4 is also HDAC independent.

The ARMs, ACR, and ZFs mediate repression of reporter gene transcription in yeast and/or HeLa cells. F1 (aa 1 to 434), the N-terminal Su(s) fragment that exhibits repression activity in both reporter gene expression assays, contains the ARMs, ACR, and ZFs. Since these motifs are required for the negative regulatory activity of Su(s) in vivo, we sought to determine whether these domains are required to repress reporter gene transcription. Thus, we introduced deletions or mutations into F1 coding sequences, subcloned the resulting DNA fragments into the appropriate Gal4 expression vectors, and tested the mutated proteins in the yeast and HeLa cell assays. As shown

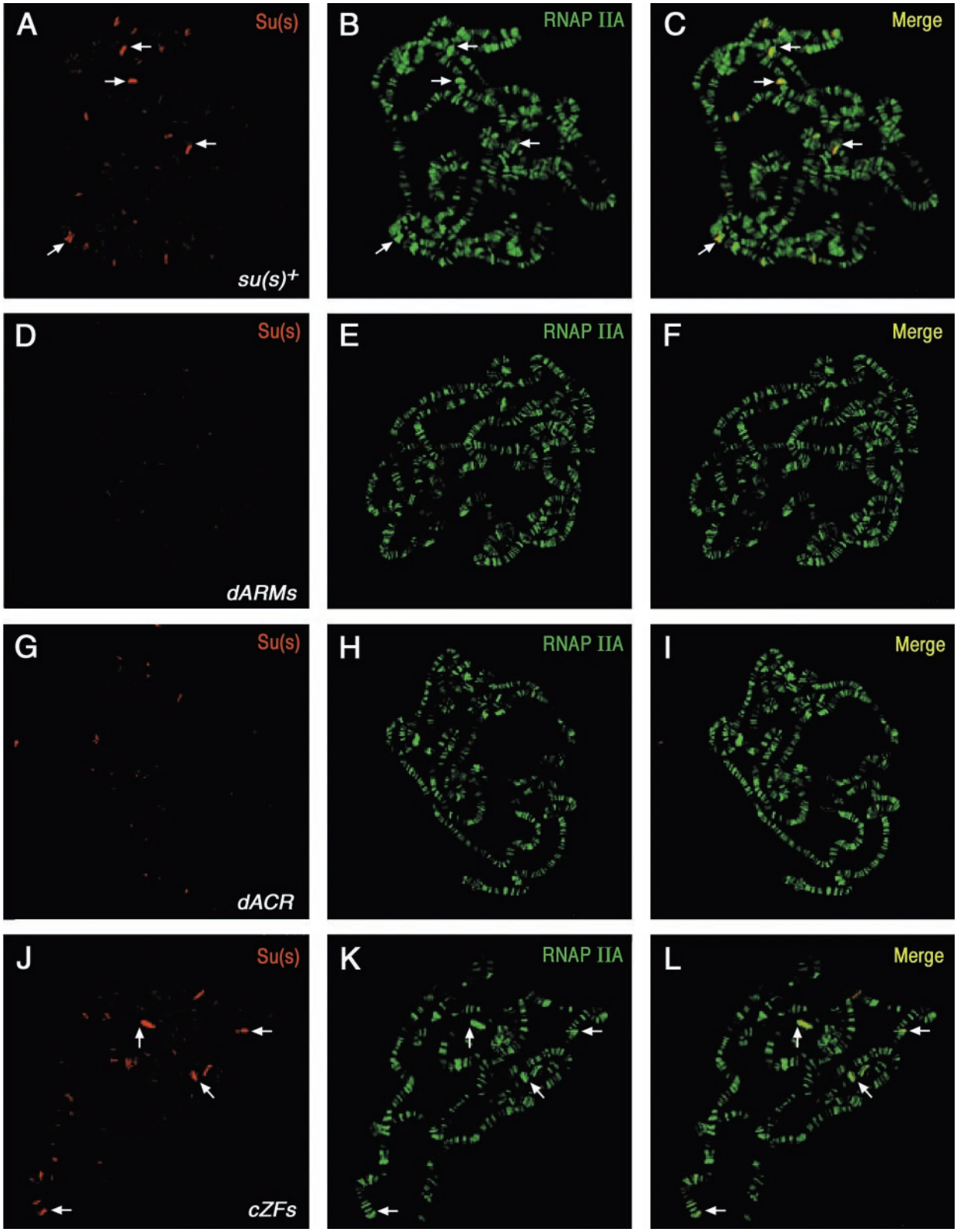

FIG. 4. The dARMs and the dACR mutant Su(s) proteins do not stably associate with polytene chromosomes. Chromosome squashes from third-instar larvae were incubated with rabbit anti-Su(s) and goat anti-RNAP IIA antibodies and visualized by indirect immunofluorescence and digital imaging (see Materials and Methods). Chromosomes were prepared from the following genetic backgrounds: *su(s)*$^+$ (A to C), *su(s)*$^-$ *[dARMs]* (D to F), *su(s)*$^-$ *[dACR]* (G to I), and *su(s)*$^-$*[cZFs]* (J to L). Anti-Su(s) is shown as red, and anti-RNAP IIA signal is shown as green. Merged red and green signals of similar intensity appear as yellow. The arrows indicate several sites of Su(s)-RNAP IIA colocalization.

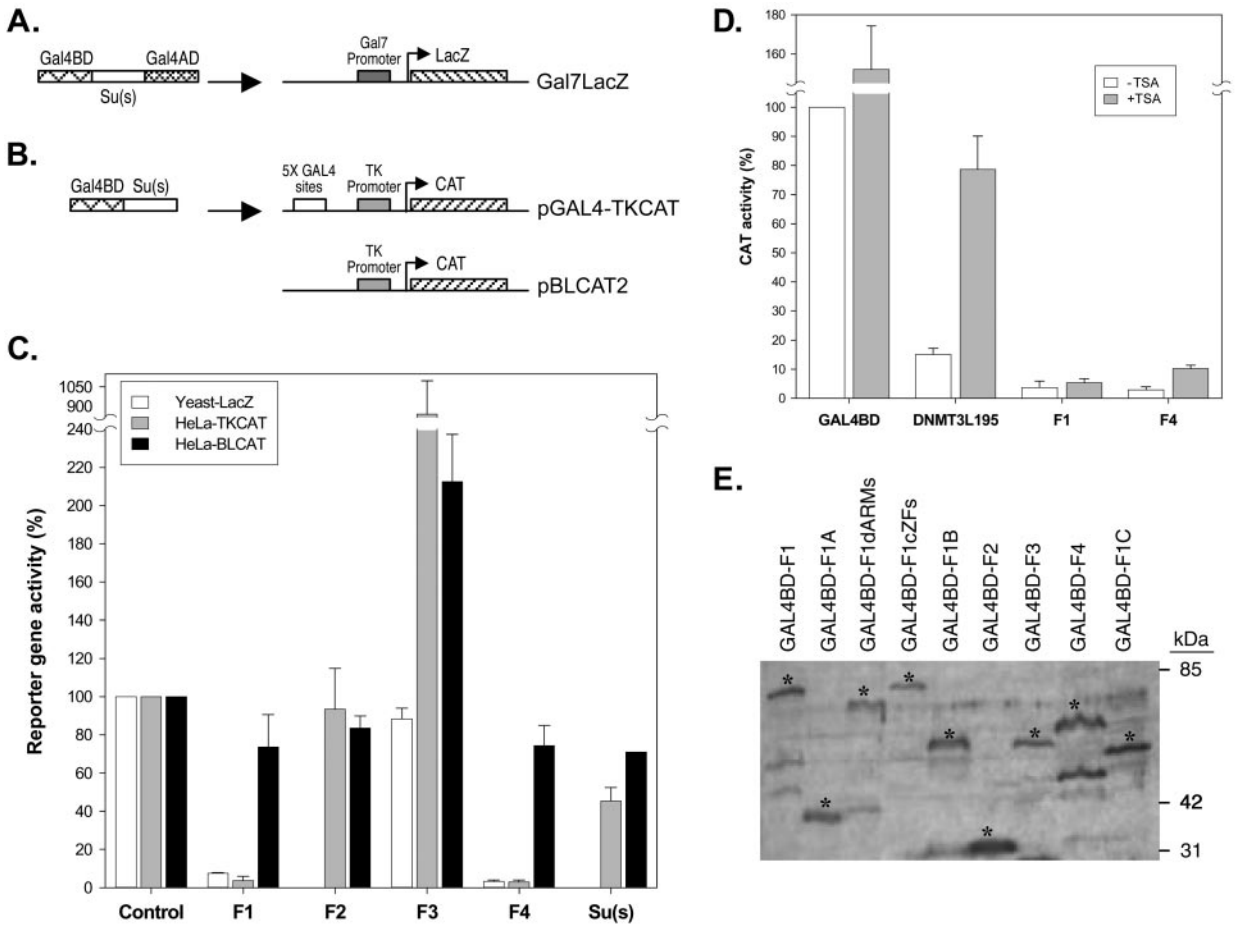

FIG. 5. The N- and C-terminal regions of Su(s) exhibit strong transcriptional repression activity in yeast and HeLa cells. (A) Schematic drawing of the yeast one-hybrid assay. The coding regions for the Su(s) fragments tested (F1 to F4, see Fig. 1) were fused downstream of Gal4 DNA-binding domain ($Gal4BD_{1-147}$) and upstream of Gal4 activation domain (Gal4AD) coding sequences. The resulting plasmids and a *lacZ* reporter gene were cotransformed into yeast. Expression of the *lacZ* reporter gene is controlled by the *Gal7* promoter, which requires the presence of Gal4AD or its equivalent to activate transcription. (B) Schematic drawing of the HeLa cell reporter gene expression assay. Coding sequences for the Su(s) fragments were fused downstream of the $Gal4BD_{1-95}$ coding region. These plasmids and a *CAT* reporter plasmid were cotransfected into HeLa cells. The *CAT* reporter gene in the test plasmid (pGAL4-TKCAT) is expressed constitutively under the control of *HSV TK* promoter, which is positioned downstream of five Gal4 DNA-binding sites. The reporter gene of the control plasmid (pBLCAT) lacks Gal4 DNA-binding sites. (C) The reporter gene activity observed in the presence of each Gal4-Su(s) fusion plasmid was compared to the empty Gal4 vectors. (D) Repression activity of F1 and F4 in HeLa cells (pGAL4-TKCAT reporter gene) in the presence or absence of 100 nM TSA (an HDAC inhibitor), added 24 h posttransfection. Each datum point in panels C and D is the mean from three independent experiments, except that full-length Su(s) was tested by using the reporter pBLCAT2 only once. (E) Western blot analysis of total protein samples isolated from transfected HeLa cells. Su(s) protein derivatives were detected by probing with polyclonal antibodies raised against Gal4 aa_{1-147} and visualized by enhanced chemiluminescence. The position of each Gal4-Su(s) fusion protein is indicated by an asterisk. The positions of molecular mass markers are indicated on the right side of the figure.

in Fig. 6, fragment F1B (aa 135 to 434), which contains both the ARMs and ZFs but not the ACR, repressed transcription 81-fold in yeast and 12-fold in HeLa cells. F1 with both ARMs deleted (F1dARMs) repressed transcription only threefold in both assays, whereas F1 with the ZFs mutated (F1cZFs) retained strong repression activity. These results indicate that the ARMs mediate a substantial component of the repression observed in both assays. Fragment F1A (aa 1 to 134), which contains the ACR, did not significantly repress reporter gene expression in yeast but repressed the reporter gene 10-fold in HeLa cells. Consistent with this, F1 with the ACR deletion (F1dACR) retained strong repression activity in yeast (68-fold) but exhibited reduced repression activity in HeLa cells (5-fold). Thus, the ACR is not involved in repression in yeast but contributes to the repression observed in HeLa cells. F1C (aa 294 to 434), which contains the ZFs, repressed transcription 12- and 8-fold in yeast and HeLa cells, respectively. This suggests that a repression domain may reside in this small fragment. However, F1 with mutated zinc fingers (F1cZFs) retained strong (16-fold) repression activity in both assays. Thus, the ZFs appear to have an independent repression activity that may be masked in these assays in the context of F1, which contains multiple repression domains.

Su(s) inhibits the transcriptional induction of a transgenic v^k construct. In a previous study (15), we generated several transgenic derivatives of the v^k allele that have a single *412*

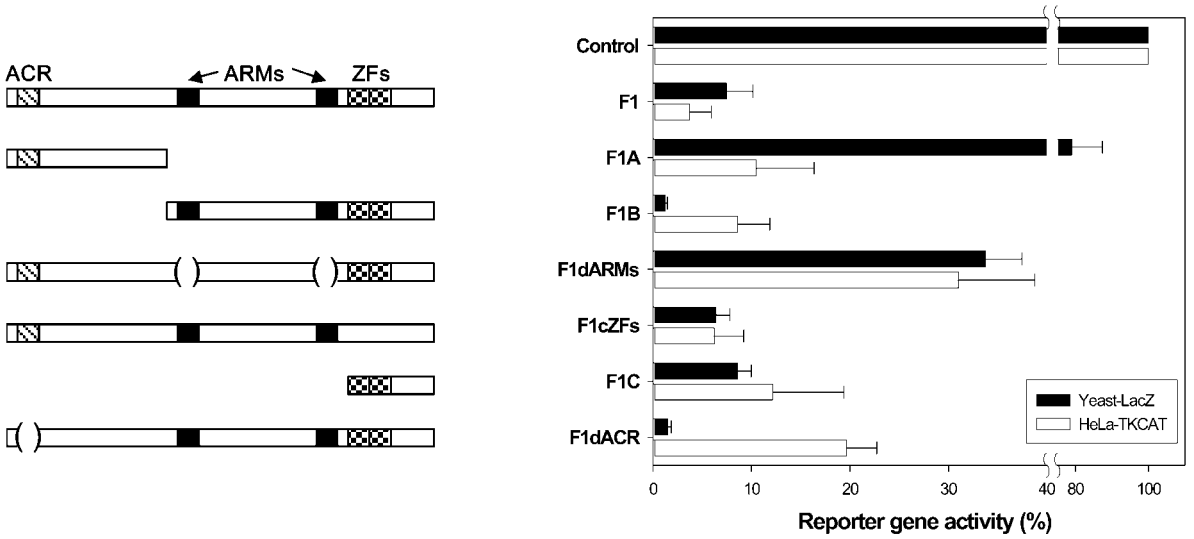

FIG. 6. The ARMs and ACR mediate reporter gene repression. The repression activity of various Gal4-Su(s)F1 (aa 1 to 434) derivatives was evaluated in yeast and HeLa cells as described in Fig. 5. The Su(s) fragments tested, shown to the left of the graph, were as follows: F1A, aa 1 to 134; F1B, aa 134 to 434; F1dARMs, F1 with both ARMs deleted; F1cZF, F1 with both ZFs mutated; and F1C, aa 295 to 434; F1dACR, F1 with the ACR deletion. Each datum point is the mean from three independent experiments. The protein levels of some of these Su(s) derivatives are shown in Fig. 5E.

long terminal repeat (LTR) in the first exon, at the same position and in the same orientation as the full-length *412* insertion of v^k. These transgenes produce pre-mRNA containing antisense LTR sequences, which are inefficiently removed by splicing at sites near the LTR ends (Fig. 7A). Cryptic 5′ and 3′ splice sites are used in splicing the LTR from the pre-mRNA of the transgene *Mtn* v^k *LTR*. However, the LTR is removed from transcripts of a second transgene (*Mtn* v^k *LTR 5′con*) by splicing from a consensus 5′ splice site, introduced by site-specific mutagenesis and located at the upstream boundary of the insertion, to the same cryptic 3′ splice site mentioned above. This consensus 5′ splice site does not improve the efficiency of splicing the LTR out of the RNA. Most likely this is because the 5′ splice site is recognized at multiple stages of splicing, and whereas the consensus sequence promotes an early recognition step, it may not be optimal for one of the later steps. However, as we observed previously (15) and as will be demonstrated below, the presence or absence of the consensus 5′ splice site at the upstream boundary of the insertion determines whether Su(s) regulates production of RNA from the transgene.

The *Mtn* promoter, which drives expression of these transgenes, can be induced by growing flies on food containing copper. Thus, in view of evidence from the reporter gene assays that Su(s) regulates transcription, we designed experiments to determine how the *su(s)* genotype affects the transcriptional induction of these two transgenes. (Flies were grown under standard conditions in our previous analysis.) In the first set of experiments, transgenic adult flies were transferred to media that were supplemented with increasing amounts of copper sulfate (0 to 0.8 mM). After 24 h, RNA was isolated from these flies and used in Northern blot analysis. The results of representative experiments are shown in Fig. 7B and C. We found that the level of *Mtn* v^k *LTR 5′con* RNA increased, as expected, over an ~10-fold range in both *su(s)*$^+$ and *su(s)*$^-$ backgrounds. In contrast, the level of *Mtn* v^k *LTR* RNA was consistently very low in the *su(s)*$^+$ background, increasing only slightly (about twofold) in the presence of 0.4 and 0.8 mM copper. In the *su(s)*$^-$ background, the uninduced level of *Mtn* v^k *LTR* RNA was about fivefold higher than in the *su(s)*$^+$ background. The *Mtn* v^k *LTR* RNA level increased further upon induction to a level that was comparable to induced *Mtn* v^k *LTR 5′con* RNA (Fig. 7B). The level of endogenous *Mtn* RNA, an internal control for the induction, increased as expected in all of the samples analyzed (Fig. 7B).

To examine RNA levels after shorter induction times, we analyzed the time course of induction of these transgenes in both *su(s)* wild-type and mutant backgrounds. To do this, we starved flies for 2 h before transferring them to media containing 0.5 mM copper sulfate for various amounts of time (between 1 and 4 h). Uninduced control flies were starved and placed on medium with no added copper. RNA samples isolated from these flies were analyzed on Northern blots, and the data are summarized in Fig. 8. We found that the induction profiles for *Mtn* v^k *LTR 5′con* were indistinguishable in wild-type and *su(s)* mutant backgrounds (Fig. 8A). RNA levels were the same in the uninduced samples and after a 1-h induction. The level of RNA was elevated at 2 h and increased further after 4 h of induction (to a maximum of 10- to 20-fold relative to the uninduced controls). In contrast, the uninduced *Mtn* v^k *LTR* RNA level was consistently very low in a *su(s)*$^+$ background and increased slightly (two- to fivefold) after 2 h of induction to a level similar to that observed in Fig. 7B (data not shown). There was not a significant increase beyond this level after 4 or 6 h of induction (Fig. 8B and data not shown). In the *su(s)*$^-$ background, the uninduced level of *Mtn* v^k *LTR* RNA was elevated relative to the *su(s)*$^+$ background, and it increased further with induction. Endogenous *Mtn* RNA was

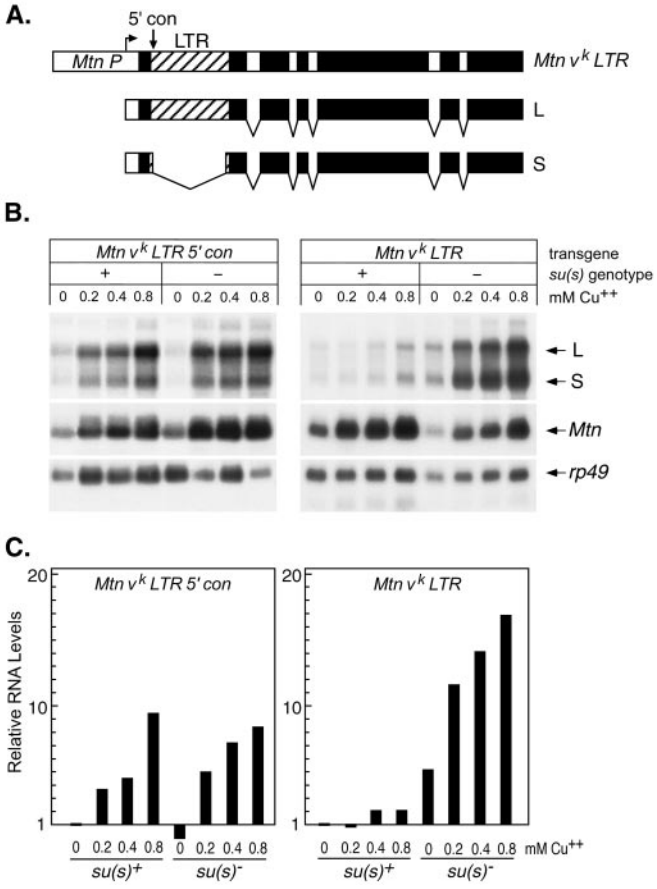

FIG. 7. Su(s) inhibits induction of *Mtn v^k LTR*, but not *Mtn v^k LTR 5′con*, over a range of copper sulfate concentrations. (A) Schematic drawing of *Mtn v^k LTR*. The diagonal bar indicates 412 LTR sequences that are inserted in the antisense orientation relative to *v* sequences. The black bars indicate *v* exons. The primary transcripts are spliced to generate a long mRNA (L) that contains the LTR and a short (S) mRNA with LTR sequences spliced out. The splicing patterns are shown below the drawing of *Mtn v^k LTR*. The position of the consensus splice site in *Mtn v^k LTR 5′con* is indicated by the vertical arrow. *MtnP* denotes the *Mtn* promoter and 5′-untranslated leader fragment. The transcription start site is indicated by the arrow in *Mtn* sequences. (B) Representative Northern blots of RNA isolated from copper-induced *Mtn v^k LTR* and *Mtn v^k LTR 5′con* flies. Each lane contains 2 μg of poly(A)$^+$ mRNA isolated from flies after feeding on media containing the various amounts of copper sulfate (Cu^{2+}, indicated above the lanes) for 24 h. The blots were sequentially probed with *v*, *Mtn*, and *rp49* probes. Arrows on the right side of the blots indicate the positions of transcripts produced by the transgenes (L and S), *rp49*, and the endogenous *Mtn* gene. (C) Graphical representations of the data in panel B. A phosphorimager was used to quantitate RNA levels, and the internal control *rp49* was used to correct for loading differences. The amount of RNA produced each transgene in the *su(s)*$^+$ background with no added Cu^{2+} was assigned a value of 1 and used as the reference point for the other RNA samples.

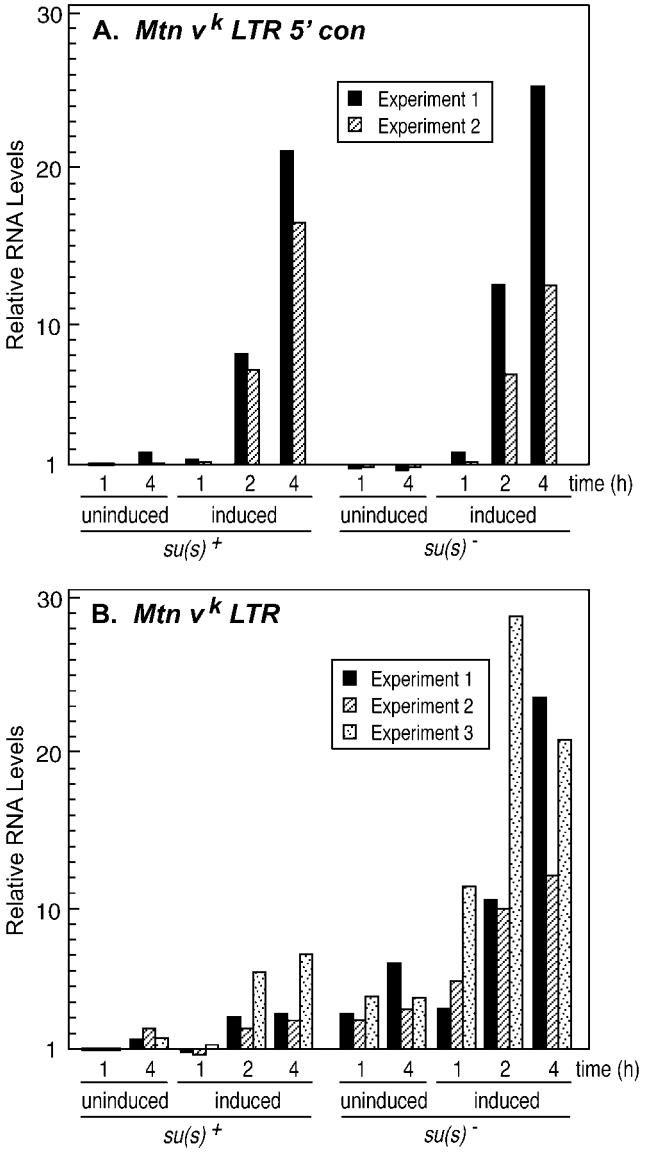

FIG. 8. Effect of *su(s)* on the time course of *Mtn v^k LTR 5′con* and *Mtn v^k LTR* induction. Graphical representation of the results of Northern blot analysis of RNA isolated from transgenic flies at various times (0 to 4 h) after transfer of adult flies to medium with no added copper (uninduced) or to medium containing 0.5 mM copper sulfate (induced). As described in Fig. 7, the amount of RNA produced each transgene in the *su(s)*$^+$ background with no added Cu^{2+} was used as the reference point for comparing the other RNA samples. (A) The *Mtnv^kLTR5′con* induction profile is the same in *su(s)*$^+$ and *su(s)*$^-$ backgrounds. (B) In the *su(s)*$^+$ background, *Mtn v^k LTR* induction is impaired, i.e., RNA levels are low (data not shown) and increase slightly after induction.

induced to comparable levels in all of the samples (data not shown). Together, the results of Fig. 7 and 8 indicate that the Su(s)-dependent regulatory pathway impairs the transcriptional induction of *Mtn v^k LTR* but not *Mtn v^k LTR 5′con*. These data support the hypothesis that Su(s) acts at the transcription level and suggest a link between the Su(s) regulatory pathway and the assembly of RNA processing components, presumably on nascent RNA.

DISCUSSION

Role for Su(s) in transcription. Our lab initially proposed that Su(s) regulates RNA stability in a manner that is connected to splicing complex assembly (15). However, the data

presented in the present study provide strong evidence that Su(s) regulates transcription or a process that is mechanistically coupled to transcription. First, we have shown that structural motifs, which mediate the negative regulatory activity of Su(s) in vivo, also mediate repression of reporter gene expression. Second, we demonstrated that the repression activity in these assays depends on the Su(s) polypeptides being tethered to the promoter region. Third, we demonstrated that Su(s) blocks the transcriptional induction of the *Mtn v^k LTR* transgene. Furthermore, recent unpublished findings from our lab provide additional support for the view of Su(s) as a transcription regulator. We have identified one site of Su(s) localization on polytene chromosomes as the *Sgs4* locus and shown that expression of this gene is leaky, i.e., a low level of RNA is produced prematurely, in a *su(s)* mutant background (Bai et al., unpublished). Furthermore, we determined that Su(s) is recruited to the vicinity of certain heat shock loci during heat shock (unpublished observations).

It is possible that Su(s) negatively regulates transcription elongation and thus induces premature termination of v^k and *Mtn v^k LTR* transcription but does not affect transcription of *Mtn v^k LTR 5′con* in this way. Transcription elongation and RNA processing are intimately coupled in vivo (7, 31), and recent evidence indicates that promoter proximal splice sites enhance transcription (16). Thus, Su(s) might play a role in arresting the synthesis of aberrant transcripts with defects in splicing complex assembly or in coordinating transcription and splicing complex assembly. Consistent with this view, mutation of *su(s)* alters the level and distribution of Ubx protein produced by bx^1 allele in haltere imaginal disks (25). Perhaps Su(s) is involved in maintaining the tightly regulated tissue-specific splicing and/or expression pattern of this allele, which contains microexons embedded within an unusually large intron. However, some of the wild-type targets of Su(s), for example, *Sgs4*, lack introns. Therefore, Su(s) activity is not always connected to splicing complex assembly.

An alternative explanation for the effect of Su(s) on *Mtn v^k LTR* is that Su(s) is involved in terminating transcription of *412*, with Su(s)-mediated regulation depending on the proximity of termination elements in the LTR and nearby splicing elements. The LTR contains elements that regulate polyadenylation and, presumably, transcription termination, which is coupled to 3′-end processing (32). Because of the orientation of the LTR, its polyadenylation signal is not expected to be recognized in *Mtn v^k LTR* pre-mRNA. However, there might be one or more terminator elements in the LTR whose function is not orientation dependent, and such elements could mediate poly(A)-independent termination events that involve Su(s).

Interestingly, recent comparative analysis of sequenced eukaryotic genomes has revealed that the region including aa 258 to 601 of Su(s) is related to the 30-kDa polyadenylation factor subunit (CPSF-30). This region, which includes the ZFs, apparently comprises a conserved ancestral domain (KOG1040; http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and, based on its presence, Su(s) has been assigned to an orthologous group that includes CPSF-30 and a subset of the other CCCH ZF proteins. Su(s) and the *Drosophila* homolog of CPSF-30 (dCPSF-30/Clp) are the only fly proteins in this group. The human proteins in this putative protein family are hCPSF-30 and KIAA1064 related proteins, which are similar in size to Su(s).

Thus, Su(s) and CPSF-30 may function in similar processes. At present, the specific molecular function of CPSF-30 is unknown; however, the CPSF complex is believed to associate with the promoter region and is needed for processing the 3′ end of nascent transcripts, which in turn promotes transcription termination (31, 32). Efficient termination promotes subsequent rounds of reinitiation. Su(s) could be involved in one or more of these processes.

Autoregulatory activity of Su(s). Our analysis of ARM and ZF mutants revealed that Su(s) negatively regulates it own expression, as indicated by the autoregulation, or failure thereof, of wild-type and mutant genomic *su(s)* transgenes. Previously, Turnage et al. (38) showed that ectopically expressed Su(s) reduces the steady-state level of RNA produced by a *Gal4*/*UAS*-driven *su(s)* cDNA transgene, which lacks all of the endogenous *su(s)* regulatory elements. It is conceivable that the inhibition of cDNA and genomic transcripts occurs by different mechanisms, both involving Su(s). For example, transcripts from the *su(s)* cDNA transgene might have been affected because Su(s), produced under Gal4 control, was present at unusually high levels or because of the unusual structure of the primary transcript generated by the *su(s)* cDNA. On the other hand, autoregulation of the genomic transgene, reported here, was observed under conditions in which Su(s) was expressed at nearly normal levels and presumably occurs in response to normal regulatory signals. However, it is possible that Su(s) negatively regulates both types of transgenes by the same mechanism, which depends on sequences within the transcribed region, basal regulatory elements, or some other common feature of the cDNA and genomic *su(s)* transgenes.

Functional domains of Su(s). (i) ARMs. These experiments have identified several repression domains that may be functionally redundant in certain contexts. The ARMs are clearly important for Su(s) function. We found that deletion of both ARMs led to substantially higher levels of v^k and *su(s)* RNA in vivo, destabilized the polytene chromosome association of Su(s), and derepressed reporter gene transcription in both yeast and HeLa cells. Since the ARMs are required for the in vitro RNA-binding activity of Su(s) (38), these results suggest that negative regulation of transcription by Su(s) depends on RNA binding. One possible mechanism whereby the ARMs might function in transcriptional repression is by binding to the nascent pre-mRNA and promoting the formation of an RNA structure that leads to the pausing or arrest of transcription elongation. Another possibility is that Su(s) is part of a repressor complex, containing a structural RNA that interacts with the ARMs. For example, the 7SK small nuclear RNA and U1 snRNA have been recently shown to bind to transcription factors and modulate their activity (22, 30, 42). The identification of cellular components that interact with the ARMs will be crucial for understanding how this domain contributes to repression.

The results of our analysis of the polytene chromosome localization of Su(s) ARM deletion derivatives differ somewhat from the data reported in a previous study of ectopically overexpressed Su(s) (38). In the present study, we found that deletion of either ARM1 or ARM2 did not significantly affect the

polytene chromosome association of Su(s), whereas the ARM double deletion greatly decreased the binding of Su(s) to chromosomes. These results are consistent with our finding that the single ARM deletions, in contrast to the double deletion mutant, do not significantly affect v^k and *su(s)* RNA levels. In the earlier, ectopic-expression experiments, Su(s) without ARM1 exhibited no detectable binding to polytene chromosomes. Su(s) without ARM2 exhibited stronger than normal binding, whereas Su(s) lacking both ARMs exhibited substantially reduced binding to chromosomes. The reasons for the discrepancies between these two sets of experiments—the behavior of the dARM1 protein in particular—are unclear. The differences may be related to the variations in experimental conditions or in the amount of Su(s) protein produced by the transgenes. However, the results obtained here, utilizing the normal *su(s)* promoter to drive normal levels of Su(s) are more likely to be biologically relevant.

(ii) ACR. We have presented here the first evidence that the ACR contributes to the normal activity of Su(s). Deletion of this region led to a twofold increase in v^k and *su(s)* RNA, destabilized Su(s) binding to polytene chromosomes, and derepressed reporter gene transcription in HeLa cells but not in yeast. Many proteins involved in transcription regulation have acidic domains. For example, the acidic domain in VP16, which was shown to bind to TFIIH, is capable of activating transcription at some promoters but inhibiting transcription at other promoters (8, 41). The acidic domain in E2F1 was shown to be important for Rb binding (21), and the acidic domain of the *Drosophila* RNAP II largest subunit was shown to bind TBP and TFIIB (26, 40). These findings suggest that the ACR may constitute a protein-protein interaction domain, mediating interactions between Su(s) and other transcription components. Perhaps the ACR interacts with a regulatory component that is present in HeLa cells but missing in yeast.

(iii) ZFs. Mutation of the two CCCH-type ZFs (CCCH-ZFs) substantially increased *su(s)* RNA levels but produced a barely detectable effect on the v^k RNA level. This suggests that Su(s) exists in different conformational states or complexes at these two genes. The ZF mutations had no apparent effect on the chromosomal association of Su(s), indicating that these motifs are important at a step subsequent to binding. Interestingly, we were only able to recover two ZF mutant transformant lines, and both of these exhibited lower viability in the *su(s)*-null mutant background than *su(s)*-null flies with no transgene (J. M. Tedesco and L. L. Searles, unpublished data). This implies that cZFs mutant protein may interfere with normal Su(s)-associated regulatory processes. The function of CCCH-type zinc fingers is unclear. Although they are not required for the in vitro RNA-binding activity of Su(s) (38), CCCH-ZFs are required for the RNA-binding activity of TTP (23). A CCCH-ZF mediates protein-protein interactions in PIE-1 (33).

(iv) Other candidate regulatory domains. Our reporter gene expression assays revealed the presence of several other Su(s) regions that are capable of repressing, activating or enhancing transcription. Of particular interest is the finding that the Su(s) C-terminal region, like the N-terminal region, exhibited strong repression activity in both reporter gene assays. The C-terminal Su(s) region has not been extensively analyzed. It exhibits low affinity in vitro RNA-binding activity (K_d of 25 nM) (37). Sequence homology searches have revealed no conserved functional domains in this region, but amino acid composition analysis indicates that the C-terminal region may contain a glycine and proline-rich domain (GP). Chen et al. (11) demonstrated that a GP domain in the *Drosophila* transcriptional corepressor Groucho (Gro) directly mediates Gro repression activity by recruiting HDACs. Only a portion of the repression activity of the C-terminal Su(s) fragment (F4) appeared to be HDAC-dependent. However, it is possible that the HDAC-dependent component involves the GP-rich region in F4. The biological relevance of these potential regulatory domains must be determined through future in vivo studies.

ACKNOWLEDGMENTS

We thank D. Dasher for initiating the *Mtv^{k}LTR* induction experiments, A. Greenleaf for providing the anti-RNAP IIA antibody, and T. K. Blackwell and P. Peterson for providing the plasmids for HeLa cell assays. We thank W. Marzluff, M. Peifer, and J. Sekelsky for providing helpful comments on the manuscript.

This study was supported by grant MCB-0111821 from the National Science Foundation.

REFERENCES

1. **Aapola, U., I. Liiv, and P. Peterson.** 2002. Imprinting regulator DNMT3L is a transcriptional repressor associated with histone deacetylase activity. Nucleic Acids Res. **30:**3602–3608.
2. **Ashburner, M.** 1989. *Drosophila*: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1995. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.
4. **Bai, W.** 2000. Immunofluorescence analysis of suppressor of sable *su(s)* protein localization on larval salivary gland polytene chromosomes of *Drosophila melanogaster*. Ph.D. thesis. Department of Biology, University of North Carolina, Chapel Hill.
5. **Barabino, S. M., W. Hubner, A. Jenny, L. Minvielle-Sebastia, and W. Keller.** 1997. The 30-kD subunit of mammalian cleavage and polyadenylation specificity factor and its yeast homolog are RNA-binding zinc finger proteins. Genes Dev. **11:**1703–1716.
6. **Batchelder, C., M. A. Dunn, B. Choy, Y. Suh, C. Cassie, E. Y. Shim, T. H. Shin, C. Mello, G. Seydoux, and T. K. Blackwell.** 1999. Transcriptional repression by the *Caenorhabditis elegans* germ-line protein PIE-1. Genes Dev. **13:**202–212.
7. **Bentley, D.** 1999. Coupling RNA polymerase II transcription with pre-mRNA processing. Curr. Opin. Cell Biol. **11:**347–351.
8. **Berger, S. L., W. D. Cress, A. Cress, S. J. Triezenberg, and L. Guarente.** 1990. Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16: evidence for transcriptional adaptors. Cell **61:**1199–1208.
9. **Burnette, J. M., A. R. Hatton, and A. J. Lopez.** 1999. *trans*-Acting factors required for inclusion of regulated exons in the Ultrabithorax mRNAs of *Drosophila melanogaster*. Genetics **151:**1517–1529.
10. **Carballo, E., W. S. Lai, and P. J. Blackshear.** 1998. Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. Science **281:**1001–1005.
11. **Chen, G., J. Fernandez, S. Mische, and A. J. Courey.** 1999. A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in *Drosophila* development. Genes Dev. **13:**2218–2230.
12. **D'Avino, P. P., S. Crispi, L. C. Polito, and M. Furia.** 1995. The role of the BR-C locus on the expression of genes located at the ecdysone-regulated 3C puff of *Drosophila melanogaster*. Mech. Dev. **49:**161–171.
13. **Deplus, R., C. Brenner, W. A. Burgers, P. Putmans, T. Kouzarides, Y. de Launoit, and F. Fuks.** 2002. Dnmt3L is a transcriptional repressor that recruits histone deacetylase. Nucleic Acids Res. **30:**3831–3838.
14. **Fridell, R. A., A. M. Pret, and L. L. Searles.** 1990. A retrotransposon 412 insertion within an exon of the *Drosophila melanogaster* vermilion gene is spliced from the precursor RNA. Genes Dev. **4:**559–566.
15. **Fridell, R. A., and L. L. Searles.** 1994. Evidence for a role of the *Drosophila melanogaster* suppressor of sable gene in the pre-mRNA splicing pathway. Mol. Cell. Biol. **14:**859–867.
16. **Furger, A., J. M. O'Sullivan, A. Binnie, B. A. Lee, and N. J. Proudfoot.** 2002. Promoter proximal splice sites enhance transcription. Genes Dev. **16:**2792–2799.
17. **Geyer, P. K., A. J. Chien, V. G. Corces, and M. M. Green.** 1991. Mutations in the *su(s)* gene affect RNA processing in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA **88:**7116–7120.

18. **Heinzel, T., R. M. Lavinsky, T. M. Mullen, M. Soderstrom, C. D. Laherty, J. Torchia, W. M. Yang, G. Brard, S. D. Ngo, J. R. Davie, E. Seto, R. N. Eisenman, D. W. Rose, C. K. Glass, and M. G. Rosenfeld.** 1997. A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. Nature **387:**43–48.
19. **Higuchi, R.** 1990. Recombinant PCR. Academic Press, Inc., San Diego, Calif.
20. **Kim, N., J. Kim, D. Park, C. Rosen, D. Dorsett, and J. Yim.** 1996. Structure and expression of wild-type and suppressible alleles of the *Drosophila* purple gene. Genetics **142:**1157–1168.
21. **Kirch, H. C., B. Putzer, G. Schwabe, H. K. Gnauck, and H. Schulte-Holthausen.** 1993. Regulation of adenovirus 12 E1A transcription: E2F and ATF motifs in the E1A promoter bind nuclear protein complexes including E2F1, DP-1, cyclin A and/or RB and mediate transcriptional (auto)activation. Cell Mol. Biol. Res. **39:**705–716.
22. **Kwek, K. Y., S. Murphy, A. Furger, B. Thomas, W. O'Gorman, H. Kimura, N. J. Proudfoot, and A. Akoulitchev.** 2002. U1 snRNA associates with TFIIH and regulates transcriptional initiation. Nat. Struct. Biol. **9:**800–805.
23. **Lai, W. S., E. Carballo, J. M. Thorn, E. A. Kennington, and P. J. Blackshear.** 2000. Interactions of CCCH zinc finger proteins with mRNA. Binding of tristetraprolin-related zinc finger proteins to Au-rich elements and destabilization of mRNA. J. Biol. Chem. **275:**17827–17837.
24. **Lindsley, D. L., and G. G. Zimm.** 1992. The genome of *Drosophila melanogaster*. Academic Press, Inc., San Diego, Calif.
25. **Manoukian, A. S., H. M. Krause, and E. W. Larsen.** 1992. Modifiers of bx1 alter the distribution of Ubx proteins in haltere imaginal discs of *Drosophila*. Dev. Biol. **151:**611–616.
26. **Mason, P. B., Jr., and J. T. Lis.** 1997. Cooperative and competitive protein interactions at the hsp70 promoter. J. Biol. Chem. **272:**33227–33233.
27. **Mello, C. C., C. Schubert, B. Draper, W. Zhang, R. Lobel, and J. R. Priess.** 1996. The PIE-1 protein and germline specification in *Caenorhabditis elegans* embryos. Nature **382:**710–712.
28. **Murray, M. V., M. A. Turnage, K. J. Williamson, W. R. Steinhauer, and L. L. Searles.** 1997. The *Drosophila* suppressor of sable protein binds to RNA and associates with a subset of polytene chromosome bands. Mol. Cell. Biol. **17:**2291–2300.
29. **Nan, X., H. H. Ng, C. A. Johnson, C. D. Laherty, B. M. Turner, R. N. Eisenman, and A. Bird.** 1998. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature **393:**386–389.
30. **Nguyen, V. T., T. Kiss, A. A. Michels, and O. Bensaude.** 2001. 7SK small nuclear RNA binds to and inhibits the activity of CDK9/cyclin T complexes. Nature **414:**322–325.
31. **Orphanides, G., and D. Reinberg.** 2002. A unified theory of gene expression. Cell **108:**439–451.
32. **Proudfoot, N. J., A. Furger, and M. J. Dye.** 2002. Integrating mRNA processing with transcription. Cell **108:**501–512.
33. **Reese, K. J., M. A. Dunn, J. A. Waddle, and G. Seydoux.** 2000. Asymmetric segregation of PIE-1 in *Caenorhabditis elegans* is mediated by two complementary mechanisms that act through separate PIE-1 protein domains. Mol. Cell **6:**445–455.
34. **Rubin, G. M., and A. C. Spradling.** 1982. Genetic transformation of *Drosophila* with transposable element vectors. Science **218:**348–353.
35. **Rutledge, B. J., M. A. Mortin, E. Schwarz, D. Thierry-Mieg, and M. Meselson.** 1988. Genetic interactions of modifier genes and modifiable alleles in *Drosophila melanogaster*. Genetics **119:**391–397.
36. **Searles, L. L., and R. A. Voelker.** 1986. Molecular characterization of the *Drosophila* vermilion locus and its suppressible alleles. Proc. Natl. Acad Sci. USA **83:**404–408.
37. **Turnage, M. A.** 1998. In vitro and in vivo analysis of the suppressor of sable [*su(s)*] gene of *Drosophila melanogaster*. Ph.D. thesis. Department of Biology, University of North Carolina, Chapel Hill.
38. **Turnage, M. A., P. Brewer-Jensen, W. L. Bai, and L. L. Searles.** 2000. Arginine-rich regions mediate the RNA binding and regulatory activities of the protein encoded by the *Drosophila melanogaster* suppressor of sable gene. Mol. Cell. Biol. **20:**8198–8208.
39. **Voelker, R. A., W. Gibson, J. P. Graves, J. F. Sterling, and M. T. Eisenberg.** 1991. The *Drosophila* suppressor of sable gene encodes a polypeptide with regions similar to those of RNA-binding proteins. Mol. Cell. Biol. **11:**894–905.
40. **Xiao, H., J. D. Friesen, and J. T. Lis.** 1994. A highly conserved domain of RNA polymerase II shares a functional element with acidic activation domains of upstream transcription factors. Mol. Cell. Biol. **14:**7507–7516.
41. **Xiao, H., A. Pearson, B. Coulombe, R. Truant, S. Zhang, J. L. Regier, S. J. Triezenberg, D. Reinberg, O. Flores, C. J. Ingles, et al.** 1994. Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53. Mol. Cell. Biol. **14:**7013–7024.
42. **Yang, Z., Q. Zhu, K. Luo, and Q. Zhou.** 2001. The 7SK small nuclear RNA inhibits the CDK9/cyclin T1 kinase to control transcription. Nature **414:**317–322.