12. The two *C. elegans* ORFs identified by the genome project are Z67884, encoded by T14G8.1, and U55373, which is encoded by yk72d6.3 and other cDNAs.
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20. Pull-down assays with a glutathione S-transferase (GST)–dMi-2(1653-1982) fusion protein and in vitro–translated ^{35}S-labeled Hb(2-789) protein (Promega TnT) were done following standard protocols. Binding was done at 4°C for 1.5 hours in 20 mM tris (pH 7.5), 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, and 1 mM dithiothreitol. After binding, the samples were washed eight times with binding buffer containing 300 mM NaCl. Samples were then boiled and loaded onto an SDS gel, and the dried gel was exposed for autoradiography. Hb protein bound to the GST–dMi-2(1653-1982) fusion protein but did not bind to GST alone. We found that only a small fraction of bacterially expressed GST–dMi-2 fusion protein is soluble. The same observation was made for a GST–dMi-2(4-1982) fusion protein.
21. Biotinylated probes were used for in situ hybridization on salivary gland chromosomes. Fine mapping of dMi-2 in the 76-dalton region was done by polymerase chain reaction (PCR) on single embryos from deficiency stocks as described by J. A. Knoblich and C. F. Lehner [*EMBO J.* **12**, 65 (1993)]. *dMi-2$^{4\text{-}9}$*, *l(3)76BDd^{3}*, *l(3)76BDd^{4}*, *l(3)76BDo^{1}*, *l(3)76BDo^{2}*, *l(3)76BDo^{4}*, *l(3)76BDo^{5}*, *l(3)76BDs^{1}*, *l(3)76BDs^{2}*, and *l(3)76BDt^{1}* were all ethylmethane sulfonate (EMS)–induced [E. B. Lewis and F. Bacher, *Dros. Inf. Service* **43**, 193 (1968)] on the same isogenized *red e* chromosome. *dMi-2^{1}* was isolated in a different screen (J. Kennison, unpublished data); *dMi-2^{2}* and *dMi-2^{3}* are P-element–induced mutations that correspond to l(3)A154.3M3 and l(3)j3D4, respectively. Details on the mapping of these mutations can be found at www.sciencemag.org/feature/data/984280.shl and in flybase.
22. Larvae that were homozygous for *l(3)76BDd^{4}*, *l(3)76BDo^{2}*, *l(3)76BDo^{5}*, *l(3)76BDt^{1}*, *dMi-2^{4}*, *dMi-2^{5}*, and *dMi-2^{6}* were identified by the *red* marker mutation on the mutant chromosome. We isolated DNA from such larvae and amplified the genomic DNA spanning the *dMi-2* ORF from codons 26 to 1982 by PCR. Four overlapping subfragments covering this interval were amplified by PCR and subcloned into Bluescript, and two independent clones were sequenced in each case. We only found sequence alterations in the case of the *dMi-2* alleles (see text). We note that the exon or exons containing the 132-nt 5′ untranslated region and first few codons appear to be separated from the other exons by a very large intron because we were unable to amplify a genomic fragment with appropriate primers.
23. *dMi-2$^{1\text{-}7}$* were each recombined onto an *FRT2A* chromosome. Germ line clones were induced with the Flp *ovo^{D1}* system. A rescue transgene that expresses the dMi-2 protein under the control of the *armadillo* promoter (*arm-dMi-2*) was constructed by substitution of the *Pc* cDNA in *arm-Pc* [J. Müller, S. Gaunt, P. A. Lawrence, *Development* **121**, 2847 (1995)] with a full-length *dMi-2* cDNA. For the rescue test, we generated females *hs-flp/+*, *arm-dMi-2/+*, and *dMi-2^{4} FRT2A/ovo^{D1}FRT2A*. The *armadillo* promoter is strongly active in the germ line, and the *arm-dMi-2* transgene rescued the germ-cell lethality due to *dMi-2* mutations. However, *dMi-2* homozygotes or transheterozygotes carrying the transgene were not rescued to adults. Somatic clones were induced in the first larval instar with the Flp/FRT system and appropriately marked chromosomes. No *dMi-2* mutant clone tissue was found in the adult epidermis compared with wild-type control clones. This suggests that dMi-2 mutant cells either die or are eliminated because of "cell competition." Imaginal discs carrying marked *dMi-2* mutant clones were stained with antibodies to Ubx or Abd-B, but no misexpression of these genes was detected. We found that 96 hours after clone induction the *dMi-2* mutant clones were substantially smaller (often they had completely disappeared) compared with the wild-type "twin spot" clones induced by the same recombination event. The same results were obtained with several *dMi-2* alleles.
24. For double-mutant combinations of *dMi-2* with PcG genes, we used *Psc^{e24}*, *Pc^{XT109}*, *Pcl^{D5}*, and an EMS-induced hypomorphic *Pc* mutation, *Pc^{e9}*. *dMi-2^{9}* homozygotes were identified by the linked *red* marker mutation. *dMi-2* homozygous embryos were identified with balancer chromosomes carrying a LacZ marker gene. *dMi-2^{4}/Pc^{XT109}* larvae were identified with appropriately marked balancer chromosomes. Standard procedures were used for staining with antibodies to Ubx, Abd-B, and β-galactosidase.
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Requirement of RSF and FACT for Transcription of Chromatin Templates in Vitro

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Transcription of naked DNA in vitro requires the general transcription factors and RNA polymerase II. However, this minimal set of factors is not sufficient for transcription when the DNA template is packaged into chromatin. Here, a factor that facilitates activator-dependent transcription initiation on chromatin templates was purified. This factor, remodeling and spacing factor (RSF), has adenosine triphosphate–dependent nucleosome-remodeling and spacing activities. Polymerases that initiate transcription with RSF can only extend their transcripts in the presence of FACT (facilitates chromatin transcription). Thus, the minimal factor requirements for activator-dependent transcription on chromatin templates in vitro have been defined.

Intense biochemical efforts have resulted in the purification of a minimal set of factors necessary for transcription of class II genes in vitro. This minimal transcription system consisting of the general transcription factors (GTFs) TFIIB, TFIID, TFIIE, TFIIF, and TFIIH and RNA polymerase II (RNAPII) was established with assays that reconstituted accurate transcription from class II promoters on naked DNA templates (*1*, *2*). In vivo, the DNA template is organized by histones into chromatin. The minimal RNAPII transcription system cannot transcribe DNA that is packaged into chromatin (*3*). However, transcription can be reconstituted on chromatin templates with nuclear extracts in the presence of an activator, suggesting that accessory factors are present in crude nuclear extracts that facilitate RNAPII transcription from chromatin templates (*4*).

Different adenosine triphosphate (ATP)–dependent nucleosome-remodeling complexes have been isolated. The NURF (nucleosome-remodeling factor) and ACF (ATP-utilizing chromatin assembly and remodeling factor) complexes were purified from *Drosophila* nuclear extracts and have been shown to facilitate activator-dependent transcription on chromatin templates (*5*, *6*). Several other ATP-dependent chromatin remodeling enzyme complexes have been purified from different organisms: SWI/SNF from yeast, human, and *Drosophila*; RSC (remodels the structure of chromatin) from yeast; and CHRAC (chromatin accessibility complex) from *Drosophila* (*7*). Biochemical studies with the human and yeast SWI/SNF complexes have suggested that it may

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participate in transcription by assisting factor binding (*8–10*) and transcription elongation (*11*) in the context of chromatin.

All previous studies that analyzed the role of remodeling enzymes in transcription of chromatin-assembled templates used crude transcription systems. We set out to identify factors that would allow a highly defined reconstituted transcription system to transcribe purified chromatin templates. To identify factors that facilitate transcription initiation on chromatin templates, we used an abortive initiation assay (*12*), in which the RNAPII transcription machinery is given only the nucleotides necessary to form the first phosphodiester bond (*13*). This assay scores for the ability of the RNAPII transcription machinery to access promoter elements and catalyze the synthesis of the first phosphodiester bond. Initiation of transcription from chromatin templates required an activity present in a crude fraction derived from HeLa cell nuclei (Fig. 1A, lane 4). This transcription initiation was specific, because no dinucleotide was formed when RNAPII and GTFs were omitted (Fig. 1A, lane 3).

We next analyzed the crude fraction for ATP-dependent nucleosome-remodeling activity. The chromatin remodeling assay used (*14*) was adapted from Tsukiyama *et al.* (*15*). For this assay, periodically spaced chromatin templates that were assembled in *Drosophila* S-190 extract, Sarkosyl-treated, and purified by gel filtration were used (*16*). The templates contained five GAL4-binding sites upstream of the adenovirus major late promoter. This chromatin was incubated with various combinations of ATP, GAL4-VP16, and the crude fraction. The chromatin was then digested with Micrococcal nuclease (Mnase), and the products were analyzed by Southern (DNA) blotting with probes corresponding to the promoter region (promoter probe) or a region of the plasmid 1000 base pairs away from the promoter (distal probe). Remodeling was indicated by a loss of periodic nucleosome spacing. Promoter proximal nucleosome remodeling was observed with the crude fraction in an ATP- and activator-dependent manner (Fig. 1B). Furthermore, this remodeling occurred only in the vicinity of the promoter because reprobing with a distal probe revealed periodically spaced nucleosomes.

We used both the abortive initiation assay and the nucleosome-remodeling assay to purify the factor that facilitates activator-dependent transcription initiation on chromatin templates (*17*). Both activities coeluted through four chromatographic steps. The active fractions from the final step of purification (Superose 12 gel filtration column) contained two polypeptides, a 325-kD polypeptide (p325) and a 135-kD polypeptide (p135), that eluted with a native molecular mass of 400 to 500 kD, suggesting that they form a heterodimer (Fig. 1C). A similar size was estimated for the complex when it was subjected to sucrose gradient sedimentation (*18*). As shown in Fig. 1, D and E, the transcription initiation activity and the nucleosome-remodeling activity coeluted during the last step of purification. The nucleosome-remodeling activity of the purified factor is ATP-dependent (Fig. 2A) and specific for the promoter region because reprobing with a distal probe revealed regular periodic nucleosome spacing. We also found that the transcription initiation activity of RSF is nucleosome specific, because it does not stimulate transcription initiation reconstituted on naked DNA templates (*18*).

We found that addition of the remodeling factor and ATP substantially improved the regularity of bulk nucleosome spacing. Therefore, we tested the remodeling factor in a nucleosome-spacing assay (*19*, *20*) that measures the conversion of irregularly spaced chromatin into nucleosome arrays with regular periodic spacing. The substrate for this assay was chromatin formed in S-190 assembly extract with an excess of core histones in the absence of ATP, Sarkosyl-treated and purified by gel filtration. As shown in Fig. 2B, the remodeling factor in the presence of ATP converted irregularly spaced chromatin (lanes 1 to 4) into arrays of nucleosomes with periodic spacing (lanes 5 and 6). The addition of ATP alone to the irregularly spaced chromatin did not alter the nucleosome spacing (lanes 1 and 2). Because the remodeling factor also exhibits nucleosome-spacing activity, we named it RSF for remodeling and spacing factor. The nucleosome-spacing activity of RSF was similar to that reported for the *Drosophila* factors ACF (*6*) and CHRAC (*20*).

SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining of purified

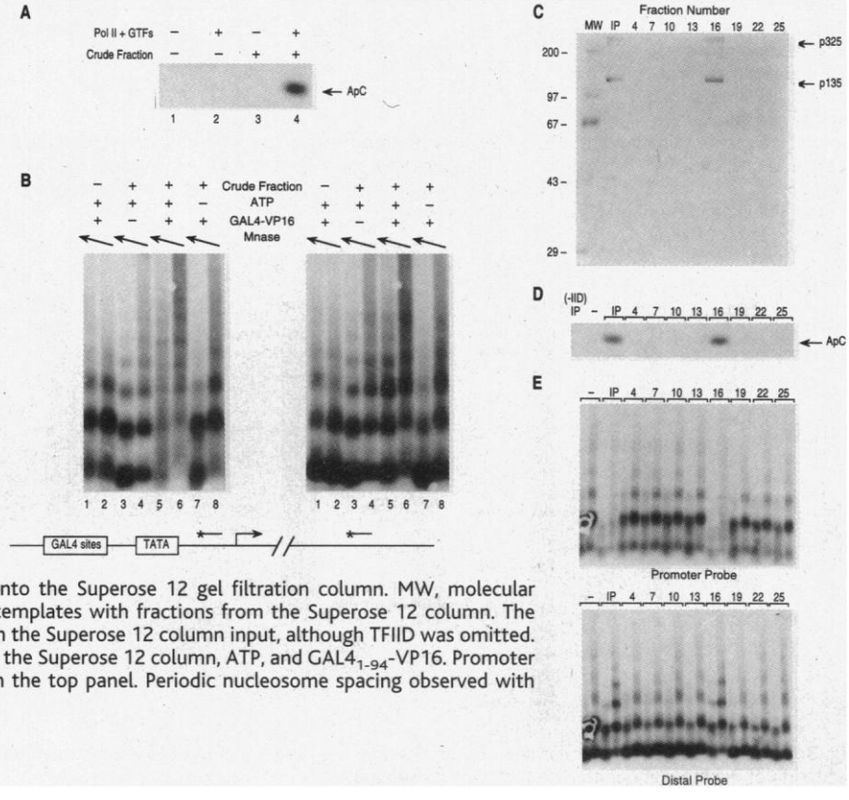

Fig. 1. (**A**) Abortive transcription initiation with purified chromatin templates, $GAL4_{1-94}$-VP16, and the reconstituted transcription system is dependent on a crude fraction (DEAE-52 fraction). Reactions were reconstituted on chromatin templates with additions as indicated. The GTFs and RNAPII used were highly purified as depicted in Fig. 3. "ApC" denotes the dinucleotide product formed. (**B**) Nucleosome-remodeling assay. Various combinations of $GAL4_{1-94}$-VP16, ATP, and the crude fraction (DEAE-52 fraction) were incubated with purified chromatin, followed by limited Mnase digestion and Southern blot analysis with a promoter probe (left) or distal probe (right). In this as in all other experiments that use Mnase, the reaction samples were split and digested with two different concentrations of Mnase, as indicated by the arrow on the top of the panel. The location of the promoter and distal probes in relation to the GAL4 DNA-binding sites and TATA box is shown below. (**C**) Coomassie blue staining of a polyacrylamide-SDS gel loaded with fractions derived from the last step of RSF purification (Superose 12 column). Protein molecular mass markers were loaded on the first lane, and their masses are expressed in kilodaltons on the left side of the figure. IP denotes the input sample loaded onto the Superose 12 gel filtration column. MW, molecular weights. (**D**) Abortive initiation assay on chromatin templates with fractions from the Superose 12 column. The reaction loaded in the first lane was reconstituted with the Superose 12 column input, although TFIID was omitted. (**E**) Nucleosome-remodeling assay with fractions from the Superose 12 column, ATP, and $GAL4_{1-94}$-VP16. Promoter proximal nucleosome-remodeling activity is shown in the top panel. Periodic nucleosome spacing observed with the distal probe is shown in the bottom panel.

RSF (Superose 12 column, fraction 16) revealed that it is composed of two subunits, a 325-kD polypeptide and a 135-kD polypeptide (Fig. 2C). We identified the subunits of RSF by sequencing peptides generated by in-gel tryptic digestion using microcapillary high-performance liquid chromatography (HPLC) directly coupled to an ion trap mass spectrometer (*21*). Thirty-four peptide sequences revealed that the 135-kD subunit is hSNF2h, a human homolog of *Drosophila* ISWI. hSNF2h is a newly identified member of the SNF2 family (*22*). On the basis of computer comparisons, hSNF2L is a human ISWI homolog (*23*, *24*) having 74.9% identity to *Drosophila* ISWI (Fig. 2D). The hSNF2L and hSNF2h proteins share 85.9% identity (Fig. 2D), suggesting that they have evolved from a gene duplication event. Fifteen tryptic peptide sequences revealed that there are no cDNAs that correspond to the 325-kD polypeptide deposited in the GenBank database. The 325-kD subunit may be susceptible to degradation because the sequences of peptides derived from a few minor polypeptides of about 180 kD revealed that they matched identical expressed sequence tags as the 325-kD polypeptide.

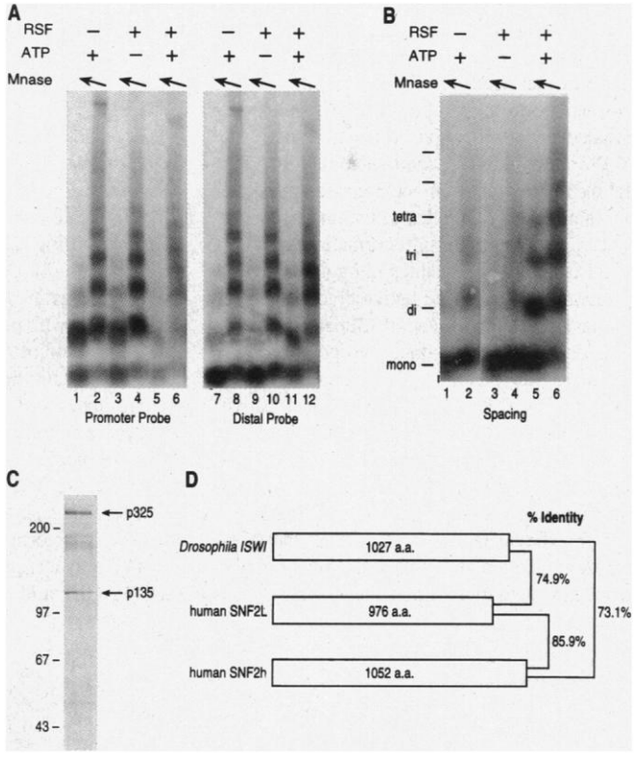

Fig. 2. The purified factor exhibits ATP-dependent nucleosome remodeling and spacing activities. (**A**) The remodeling activity of RSF is ATP-dependent. Purified chromatin templates and $GAL4_{1-94}$-VP16 were incubated with various combinations of RSF and ATP, as indicated on the figure. The reactions were then subjected to partial digestion with Mnase and Southern blot hybridization with the promoter probe (lanes 1 to 6) or distal probe (lanes 7 to 12). (**B**) RSF exhibits ATP-dependent nucleosome spacing activity. Irregularly spaced chromatin was incubated with ATP alone (lanes 1 and 2), RSF alone (lanes 3 and 4), or RSF and ATP (lanes 5 and 6) followed by limited Mnase digestion and Southern blot analysis. (**C**) Silver staining of a polyacrylamide-SDS gel containing a portion of RSF derived from the last step of purification (Superose 12). Peptide sequencing by microcapillary HPLC–ion trap mass spectrometry identified the 135-kD polypeptide as human SNF2h. (**D**) Schematic comparison of the identity between *Drosophila* ISWI, human SNF2L, and human SNF2h; a.a., amino acid.

Next, we used a reconstituted transcription system to determine whether the ability of RSF to direct transcription initiation using a reconstituted transcription system was dependent on an activator. The reconstituted transcription system used was composed of the highly purified recombinant GTFs TFIIB, TFIIE, and TFIIF and the affinity-purified multisubunit mammalian factors TFIID, TFIIH, and RNAPII (Fig. 3). In addition, the system also contained the highly purified recombinant factor GAL4-VP16 and the coactivators, PC4 and TFIIA (Fig. 3), that are minimally required for activation (*25*). The chromatin used for transcription assays was assembled in *Drosophila* S-190 extract, Sarkosyl-treated, and purified by gel filtration. SDS-PAGE followed by silver staining of the purified chromatin templates revealed that they are greater than 95% pure (Fig. 3). Transcription initiation on chromatin templates requires both RSF and an activator (Fig. 4A, lane 7). GAL4-VP16 did not promote initiation in the absence of RSF (lane 4). Also, the addition of RSF in the absence of GAL4-VP16 (lane 5) or in the presence of $GAL4_{1-94}$ (lane 6), which lacks an activation domain, did not result in initiation. Only the combination of RSF and GAL4-VP16 resulted in strong initiation (lane 7). This initiation was RNAPII-specific because no initiation product was detected in an identical reaction where GTFs were omitted (lane 8).

Next, we determined whether these initiated polymerases could elongate their transcripts. As shown in Fig. 4B (lane 9), the reconstituted system was not capable of full-length productive transcription in the presence of RSF and an activator. Under identical conditions, transcription initiation was observed (Fig. 4A, lane 7).

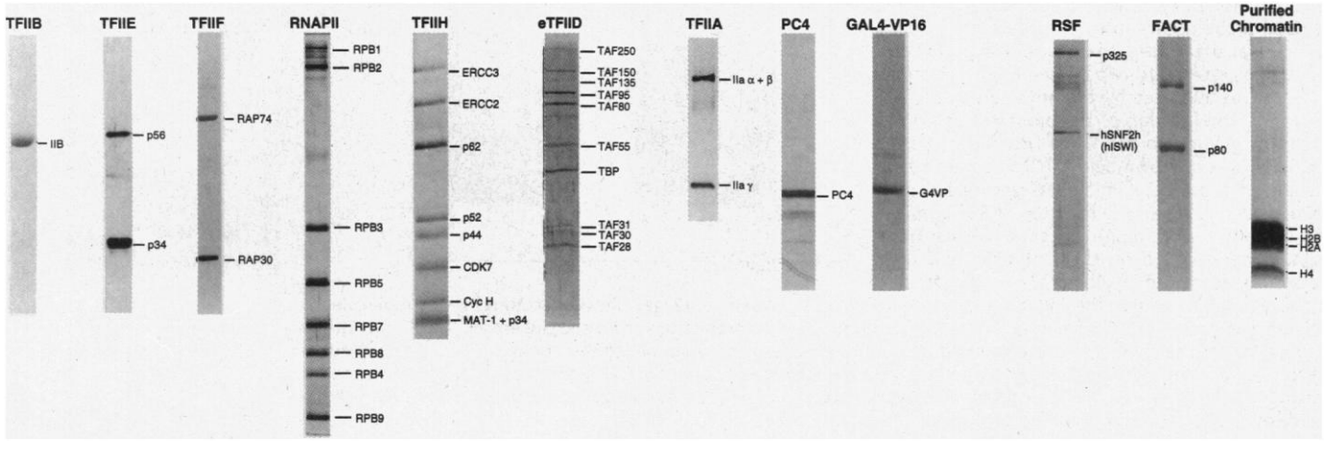

Fig. 3. Silver staining of polyacrylamide-SDS gels containing the factors used to reconstitute transcription on chromatin templates. The labels on the right side of each panel denote the factor or its subunits.

We have previously demonstrated that the failure of the reconstituted transcription system to elongate its transcripts on remodeled templates is due to nucleosome-induced stalling (*26*). Productive transcription through nucleosomes requires a factor termed FACT (facilitates chromatin transcription) (*26*). FACT is a heterodimeric factor of 140- and 80-kD polypeptides (Fig. 3), whose subunits have recently been identified (*27*). The addition of FACT in the presence of RSF and an activator facilitated the production of a 390-nucleotide (nt), full-length transcript (Fig. 4B, lane 7). However, FACT did not promote transcription in the absence of RSF, even when an activator was present (lane 4). Consistent with the results from our initiation assays, strong transcription was not observed in the absence of an activator (lanes 2 and 5) or in the presence of the $GAL4_{1-94}$ DNA-binding domain alone (lanes 3 and 6). The activator-dependent transcription observed in the presence of RSF and FACT was RNAPII-specific, because no product was detected when GTFs were omitted (Fig. 4B, lane 8).

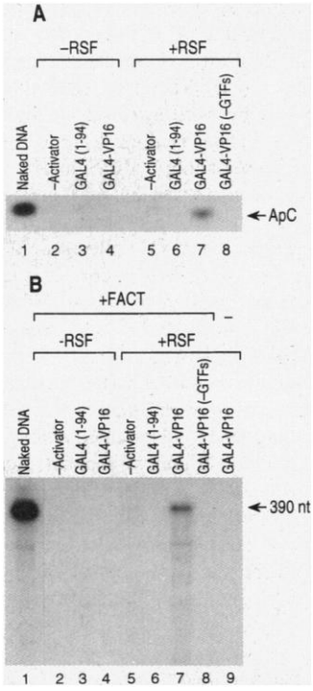

Fig. 4. Activator-dependent reconstituted transcription on chromatin templates minimally requires GTFs, RSF, and FACT. **(A)** Initiation of transcription with the reconstituted system on chromatin templates requires both an activator and RSF. The presence or absence of RSF and the addition of $GAL4_{1-94}$-VP16 or $GAL4_{1-94}$ are indicated on the figure. GTFs were omitted in lane 8. Lane 1 is transcription initiation reconstituted on naked DNA. **(B)** Productive full-length transcription on chromatin templates requires RSF and FACT. Transcription was reconstituted under the same conditions used for initiation assays, except that the reactions were provided with a nucleotide mixture sufficient to generate a 390-nt transcript. RSF, FACT, and $GAL4_{1-94}$-VP16 or $GAL4_{1-94}$ were added as indicated in the figure. GTFs were omitted from the reaction loaded in lane 8. Lane 1 is full-length transcription reconstituted on naked DNA.

This report describes a system that is capable of reconstituting both the initiation and elongation phases of transcription from chromatin templates in vitro. Previous studies that reconstituted transcription from chromatin templates used crude systems with many unidentified proteins (*3*). RSF is capable of directing activator-dependent transcription initiation on chromatin templates. We believe that RSF facilitates transcription initiation by remodeling nucleosomes in the promoter region to allow the formation of preinitiation complexes. However, the activities of RSF are not sufficient to allow polymerases to extend their transcripts. Elongation through nucleosomes requires FACT, a chromatin-specific elongation factor (*26*). Although we did not use the *Drosophila* NURF complex in our assays, we expect it will substitute for RSF. Also, because the activities of the *Drosophila* ACF complex are similar to those of RSF, it may also facilitate transcription initiation in our system. We do not believe that the SWI/SNF complex or the *Drosophila* CHRAC complex will substitute for RSF in our assays because a recent study found that these complexes could not substitute for NURF in an in vitro transcription assay (*5*).

In this study, we have defined the minimal factor requirements for activator-dependent transcription on chromatin templates. It is likely that there are other activities present in HeLa cells that will function in reconstituted chromatin transcription assays. Additional coactivators or mediator type complexes may be required for high levels of transcriptional activation. Also, histone acetyltransferase complexes may stimulate transcription from chromatin templates. The use of chromatin transcription assays will provide a more natural environment to study transcription factors in vitro.

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12. The abortive initiation and productive transcription assays were performed as described in (*26*) with the following variations: (i) The chromatin used for these assays was Sarkosyl-treated (0.05% Sarkosyl) before purification. (ii) $GAL4_{1-94}$ or GAL4-VP16 was added during the transcription reaction, not during chromatin assembly. (iii) The TFIIH used in transcription assays was purified by affinity chromatography.
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16. Chromatin was assembled with *Drosophila* S-190 assembly extract as described in (*28*) with 3 μg of supercoiled plasmid DNA and 2 μg of HeLa cell core histones in a 400-μl reaction volume. The plasmid DNA (PG5MLP) used in these studies contained five GAL4 DNA-binding sites located upstream of the adenovirus major late promoter followed by a 390-nt G-less cassette. After 4.5 hours of chromatin assembly, Sarkosyl was added to the assembly reaction to a final concentration of 0.05% and incubated for 5 min at 25°C. After Sarkosyl treatment, the chromatin was purified from assembly reaction (400 μl) with a CL4B gel filtration column as described in (*26*).
17. RSF was purified from HeLa cell nuclear pellets through five purification steps. These steps included an ammonium sulfate precipitation, two anion exchange chromatography columns, one cation exchange chromatography column, and a gel filtration column. A detailed protocol is available at www.sciencemag.org/feature/data/984132.shl
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19. The nucleosome spacing assay performed in this manuscript is a variation of the assay described in (*20*). Briefly, substrate was prepared by assembling chromatin in *Drosophila* S-190 extract as described above, although in the absence of ATP and with an excess of HeLa cell core histones. After assembly, the chromatin was treated with Sarkosyl and purified by gel filtration as described above. This irregularly spaced chromatin substrate (200 ng) was then incubated with 5 μl of purified RSF (Superose 12 fraction) in the presence or absence of 4 mM ATP in the same buffer conditions as described for the remodeling assays. Nucleosome spacing was observed after partial digestion with Mnase and Southern hybridization with the distal probe, carried out in the same conditions as the remodeling assays.
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21. About 1 to 3 pmol of RSF was run on a 10% SDS-PAGE stained with Coomassie blue, and the appropriate polypeptide bands were excised. After in gel digestion, peptide sequence information was determined by microcapillary reversed-phase chromatography [H. M. Nash *et al.*, *Curr. Biol.* **6**, 968 (1996)] coupled to the electrospray ionization source of a quadrupole ion trap mass spectrometer (Finnigan LCQ). The ion trap's online data-dependent scans allowed the automatic acquisition of a high-resolution scan to determine charge state and exact mass and tanden mass spectrometry spectra for peptide sequence information. Identification of spectra corresponding to known peptide sequences in the National Center for Biotechnology Information nr and dbest databases was facilitated with the algorithm Sequest [J. K. Eng, A. L. McCormick, J. R. Yates III, *J. Am. Soc. Mass Spectrom.* **5**, 976 (1994)], followed by manual confirmation.
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was affinity-purified from HeLa cells with a monoclonal antibody (mAb) to ERCC3 as described by G. LeRoy *et al.* [*J. Biol. Chem.* **273**, 7134 (1998)]. TFIID was affinity-purified from HeLa cells expressing hemagglutinin-tagged TATA binding protein with an mAb to hemagglutinin as described by Q. Zhou *et al.* [*Genes. Dev.* **6**, 1964 (1992)]. RNAPII was affinity-purified from HeLa cells with an mAb to RNAPII as described in (*29*). PC4 was expressed in *E. coli* and purified as described by H. Ge *et al.* [*Cell* **78**, 513 (1994)]. $GAL4_{1-94}$ and $GAL4_{1-94}$-VP16 were expressed in *E. coli* and purified as described by R. J. Reece *et al.* [*Gene* **126**, 105 (1993)]. FACT was purified from HeLa cells up to the phosphocellulose step as described in (*26*).

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Patterning of Cortical Efferent Projections by Semaphorin-Neuropilin Interactions

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Cortical neurons communicate with various cortical and subcortical targets by way of stereotyped axon projections through the white matter. Slice overlay experiments indicate that the initial growth of cortical axons toward the white matter is regulated by a diffusible chemorepulsive signal localized near the marginal zone. Semaphorin III is a major component of this diffusible signal, and cortical neurons transduce this signal by way of the neuropilin-1 receptor. These observations indicate that semaphorin-neuropilin interactions play a critical role in the initial patterning of projections in the developing cortex.

The directed growth of cortical efferent axons toward the white matter suggests that they might be guided by extracellular signals (*1*). To detect the presence and distribution of such signals, we developed a slice overlay assay that would allow us to assess the effect of local extracellular signals on axon growth and guidance. In this assay DiI-labeled cortical neurons were plated onto slices of cortex that provided a substrate for axon growth (*2*). When embryonic day 18 (E18) neurons were plated onto E18 or postnatal day 3 (P3) cortical slices, over 85% of the labeled cells had extended axons by 3 hours after plating that could be clearly scored for length and orientation (*3*). This analysis revealed marked differences in the pattern of axon outgrowth across the cerebral wall. Whereas labeled cells plated over the intermediate zone of E18 slices and over the white matter of the P3 slices did not show oriented axon outgrowth, virtually all of the cells plated over the cortical plate had an axon that was directed toward the ventricular surface (Fig. 1, A and B) (*4*). Quantitative analysis of the axons growing over the cortical plate indicated that almost 80% were directed toward the ventricular surface (ventricle) and less than 10% were directed toward the pia (Fig. 1C). Thus, an extracellular signal locally detectable in the cortical plate is responsible for the directed outgrowth of cortical axons on cortical slices.

Although the slice overlay experiments revealed that the cortical plate contains a signal that directs axon outgrowth, they did not reveal the nature and source of the signal. To determine if this signal could act at a distance to influence the cortical axon orientation, we examined whether a second slice of cortex placed next to the white matter could affect the behavior of labeled cells plated over the white matter. Cells plated over the white matter normally do not show oriented axon growth (Fig. 2, A, F, and G). When a second slice of cortex was placed immediately adjacent to the white matter, the cells plated over the white matter extended axons directed away from the second slice (Fig. 2, A, C, and G). This result was particularly striking because cells on either side of the marginal zone of the second slice had axons directed away from the marginal zone (Fig. 2, C and D). Therefore, cortical neurons are repelled by a diffusible signal present at high concentrations near the marginal zone.

Because semaphorin III (SemaIII) and its orthologs (semaphorin D and collapsin-1) have been extensively characterized as diffusible chemorepulsive molecules (*5*), and because SemaIII is expressed in the cortical plate and marginal zone both at E18 and P3 (*6*), we examined the role of SemaIII in cortical axon guidance. If SemaIII is involved in orienting axon growth in the cortical plate, then perturbation of a putative SemaIII gradient should lead to disoriented axon growth. To test this possibility, we incubated cortical slices with 293T cell–derived recombinant SemaIII (*7*) to reduce or abolish any SemaIII gradient before plating labeled cortical neurons onto the slices. In control slices (incubated with conditioned media from untrans-

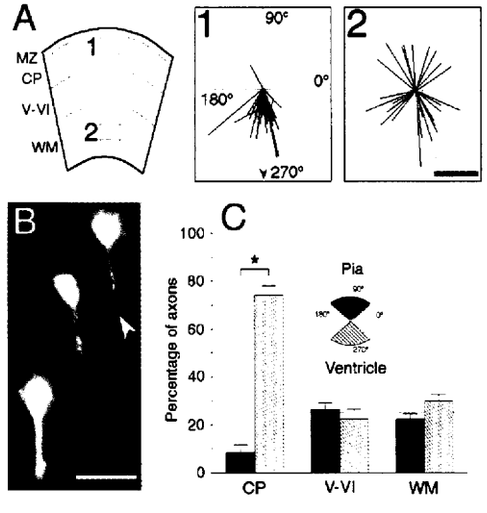

Fig. 1. A signal in the cortical plate (CP) directs axons toward the white matter (WM). (**A**) Axon orientations of E18 cortical neurons plated over the CP (box 1) or WM (box 2) of P3 cortical slices. MZ, marginal zone; V-VI, cortical layers V and VI. Bar, 25 μm. (**B**) Video image of E18 neurons growing over the CP of a P3 slice. The growth cones are directed toward the WM (arrowhead). Bar, 10 μm. (**C**) Axon orientation histograms of E18 neurons plated over P3 slices. The horizontal line in this and other histograms indicates expected distribution for random orientation. Statistically significant differences ($P < 0.001$) are indicated by an asterisk (*3*).

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