catalytic reaction into movement but rather is a response of the moving complex to an obstacle encountered on the way (*12*).

The characteristics of the strained elongation complex (shortened C~F distance, filled loose product site, sensitivity to GreB) and its exclusive tendency to convert into the dead-end state find analogy in the phenomenon of elongation arrest by eukaryotic RNA polymerase II (*13*). The arrested Pol II complex is characterized by high sensitivity to the eukaryotic transcript cleavage factor S2, the long register of cleavage, and a shortened distance between the RNA 3′ end and the front edge of the complex. By contrast, elongation-competent Pol II complexes artificially halted elsewhere in the template resemble the preferred conformation of the elongation complex in *E. coli*. These correlations strongly suggest that the model presented here for *E. coli* holds true for the mechanism of transcription elongation in eukaryotic systems.

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14. The standard template for transcription (template 2) was a polymerase chain reaction (PCR)–amplified 324-bp DNA fragment carrying the T7A1 promoter, in which the first 59 nt of the transcribed region was the same as in template 1 of (*4*). This promoter region was followed by the sequence TG and then by the sequence shown in Fig. 2 and Fig. 4B. His-tagged RNA polymerase was purified and immobilized on Ni-NTA–agarose as described (*8*). In the walking reaction for 12 samples (the experiment of Fig. 2), 20 μl of wet Ni-NTA–agarose pellets were used to immobilize 2 pmol of ribonucleic acid polymerase (RNAP), then 4 pmol of promoter-carrying fragment were added followed by incubation for 5 min at 37°C. The transcription was started by the addition of the priming dinucleotide ApU (0.2 mM), unlabeled adenosine triphosphate (ATP), cytidine 5′-triphosphate, and guanosine triphosphate (25 μM each) and was continued for 10 min at 25°C to make the 20-nt complex (EC_{20}). The reaction was stopped by resin pelleting and washing (*8*), and the RNA in the complex was extended with [α-^{32}P]uridine 5′-triphosphate (3 μl, 300 Ci/mmol, 5 min) to form 3′ end-labeled EC_{21}. Subsequent alternation of the washing and chain extension steps was used for further walking of the complex as described in (*8*). Samples were withdrawn at each walking step in the amount of one-twelfth of the total reaction mixture.
15. The template fragment for Exo III footprinting was obtained by PCR with the use of nonphosphorylated (left) and 5′-phosphorylated (right) 24-nt primers to produce a 5′-OH group in the nontemplate strand for the subsequent enzymatic phosphorylation. The product of PCR was purified from low-melting agarose gel and the nontemplate strand was labeled by T4 polynucleotide kinase and [γ-^{32}P]ATP. The 5′ terminal labeled template was used for the preparation of the stalled elongation complexes as described in (*14*). Exonuclease III (10 U) was added to the immobilized elongation complex in a volume of 10 μl (3 μl of Ni^{2+}-agarose suspension and 7 μl of transcription buffer) and incubated for 6 min at 25°C. The reaction was either stopped by addition of EDTA (50 mM) or Exo III was removed by centrifugation and washing (*14*) and fresh NTPs (250 μM each) were added in "chase" samples (Fig. 2, bottom panel). The Exo III-derived DNA products were separated by 6% denaturing polyacrylamide gel electrophoresis (PAGE).
16. GreB protein was purified as described (*4*). The cleavage reaction of RNA in the immobilized elongation complexes (EC_{52}, EC_{56}, and EC_{70-80}) was carried out in the transcription buffer in 10-μl samples. Each reaction contained ~0.3 pmol of defined ternary complex immobilized on 2 to 5 μl of Ni^{2+}-agarose suspension. GreB was added in the amounts indicated in the figures (180 pmol in the experiment shown in Fig. 5, top panel), and samples were incubated for 10 min at 25°C. For the reaction shown in Fig. 3 (bottom panel), GreB was added to the Exo III–treated complexes after the removal of Exo III protein by centrifugation and washing. All reactions were stopped by addition of EDTA (to 50 mM). RNA products were separated by 6% denaturing PAGE.
17. Because the specific activity of GreB varies substantially from preparation to preparation, the absolute levels of sensitivity to cleavage differ from experiment to experiment (compare Figs. 3 and 5). Thus, the summary data of Table 1 describe a cumulative picture of relative sensitivities of different complexes determined in several experiments.

18. We are grateful to S. Borukhov for providing the GreB protein used in these studies and to other members of the Goldfarb laboratory for helpful discussions. Supported by the NIH grant GM49242 and NSF grant MCB-9218217 (to A.G.). M.K. was supported in part by a long-term fellowship from the Human Frontier Science Program.

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Stimulation of RNA Polymerase II Elongation by Chromosomal Protein HMG-14

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The high-mobility group protein 14 (HMG-14) is a non-histone chromosomal protein that is preferentially associated with transcriptionally active chromatin. To assess the effect of HMG-14 on transcription by RNA polymerase II, in vivo–assembled chromatin with elevated amounts of HMG-14 was obtained. Here it is shown that HMG-14 enhanced transcription on chromatin templates but not on DNA templates. This protein stimulated the rate of elongation by RNA polymerase II but not the level of initiation of transcription. These findings suggest that the association of HMG-14 with nucleosomes is part of the cellular process involved in the generation of transcriptionally active chromatin.

Transcription in mammalian cells occurs in the context of chromatin, a complex of genomic DNA with histones and non-histone proteins. Consequently, the transcription of a gene can be regulated by alterations in its chromatin structure (*1*). Transcriptionally active chromatin is distinguished from inactive chromatin by alterations in protein composition and by protein and DNA modifications. Candidates for potentiators of the switch from an inactive to an active chromatin structure include proteins associated specifically with active chromatin. The HMG-14 and the closely related HMG-17 proteins are two such candidates, and a variety of experimental data couple these proteins with

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active chromatin (*2*, *3*). Each nucleosome contains two high-affinity binding sites for HMG-14 and HMG-17, which are the only known nuclear proteins that bind directly to the nucleosome core particle (*4*). However, the amount of HMG-14 and HMG-17 per nucleus is only sufficient to bind to a subset of the nucleosomes (*5*). The potential for HMG-14 and HMG-17 to regulate gene expression has also been suggested by the inhibition of myoblast differentiation upon the aberrant expression of HMG-14 (*6*). Thus, cellular differentiation may require the regulated expression of HMG-14 to control gene expression appropriately.

In spite of these findings, a causal relation between the HMG-14 and HMG-17 proteins and the transcriptional induction of chromatin templates has been unclear. Recently, it was shown that the incorporation of HMG-17 into chromatin on newly replicated DNA enhanced the transcription of genes by RNA polymerase III in a *Xenopus* egg extract assembly system (*7*). Here, we demonstrate that HMG-14 directly stimulates transcription on chromatin templates by RNA polymerase II (pol II) in the absence of DNA replication. In vivo–assembled chromatin, namely simian virus 40 minichromosomes (SV40 MCs), was used as the template for these in vitro transcription studies (*8*, *9*). In contrast to chromatin reconstituted in vitro, SV40 MCs provide nonrepressed chromatin templates that are transcriptionally competent. The initiation of transcription from the isolated SV40 MCs is both efficient and correctly regulated in comparison with the in vivo characteristics of these nucleosomal templates (*8*). Therefore, SV40 MCs provide an ideal system for the modification of the protein composition of chromatin templates to determine how distinct chromosomal proteins affect transcriptional potential.

To ascertain the effect of HMG-14 on transcription by pol II, we initially increased the amount of HMG-14 bound to the in vivo–assembled SV40 MCs. We established CV-1 cell lines, permissive for SV40 infection, that overexpressed human HMG-14 mRNA (*10*). Northern (RNA) analysis of total cellular RNA identified several clones expressing high amounts of the human HMG-14 mRNA (Fig. 1A), when compared to either the parental line or to a cell line stably transfected with vector only. The MCs isolated (*11*) from cells expressing human HMG-14 mRNA contained elevated levels of HMG-14 [Fig. 1B, MC (14)], when compared with MCs isolated from the parental CV-1 cells [MC (wt)]. These two preparations of MCs were transcribed in vitro with a pulse-chase protocol (*12*) (Fig. 2A). When the data were normalized to the internal control of transcripts from naked DNA (Ad MLP and EE_m), transcription from the SV40 MC early (LE, EE) and late [L325 (1)] promoters was increased 2.8 to 5.5 times in the presence of elevated levels of HMG-14 [MC (14) versus MC (wt)]. These data suggest that HMG-14 stimulates transcription by pol II.

The deposition of HMG-14 and HMG-17 into nucleosomes in vivo may occur during nucleosome assembly on nascent DNA (*7*). Thus, it was important to determine whether the addition in vitro of human HMG-14 to the non-replicating but transcriptionally competent SV40 MCs would result in similar transcriptional enhancement from the MCs. Purified recombinant human HMG-14 (*13*) binds tightly to SV40 MCs (*14*). Recombinant HMG-14 stimulated transcription in a dose-dependent fashion from MCs isolated from parental CV-1 cells (Fig. 2B). No effects of HMG-14 were observed upon the transcription of naked DNAs (Ad MLP DNA in all lanes and SV40 viral DNA in right two lanes, Fig. 2B). Thus, HMG-14 stimulates transcription by pol II, but only in the context of chromatin. This result indicates that HMG-14 stimulates transcription by affecting the structure of nucleosomes but not the transcriptional machinery per se. The transcriptional stimulation obtained with recombinant HMG-14 implies that the transcriptional effect of HMG-14 assembled in vivo (Fig. 2A) was due to the presence of HMG-14 and not to other proteins brought to the MCs in the cell by the increased levels of HMG-14.

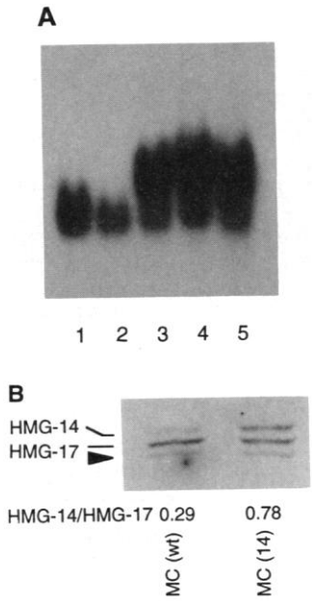

Fig. 1. (**A**) Northern blot analysis of HMG-14 mRNA from stable, overexpressing cell lines: lane 1, CV-1 cell line stably transfected with vector (pLEN); lane 2, parental CV-1 cell line; lanes 3 to 5, CV-1 cell lines stably transfected with human HMG-14 cDNA. The HMG-14 mRNA produced within the pLEN vector has a longer 3′ untranslated sequence and, therefore, mRNAs of increased size. Northern analysis of total RNA from tissue culture cells was performed by standard procedures (*19*) with ^{32}P-labeled probe generated by random priming of the human HMG-14 cDNA. (**B**) Immunoblot analysis of HMG-14 and HMG-17 from MCs isolated either from the parental cell line [MC (wt)] or from the cell line overexpressing HMG-14 mRNA [MC (14)]. HMG-14 and HMG-17 were extracted from MCs with 5% perchloric acid as described (*13*). The proteins were resolved on an SDS–15% polyacrylamide gel, transferred to Immobilon-P membrane (Millipore), and probed with antiserum to HMG-14 and HMG-17 (*24*). The ratio of HMG-14 to HMG-17 is given at the bottom. The identity of the protein band indicated by the solid triangle is not clear, possibly another isoform or a degraded product of HMG-14 or HMG-17.

To determine whether HMG-14 affects initiation or elongation, or both, of transcription on the MCs, two experiments were conducted that differed only in whether HMG-14 was added after or before the initiation process in the in vitro transcription protocol. In the first case, transcription was initiated with limiting concentrations of nucleotides (one of which was radioactive) in the absence of HMG-14. Only subsequently was HMG-14 added, along with excess unlabeled nucleotides to prevent the detection of subsequent rounds of initiation. The addition of HMG-14 at this point elevated approximately threefold the level of transcripts from the MCs of 1457 nucleotides (nt) or longer (Fig. 3A). Because HMG-14 was not added until after the transcripts were initiated, it must have promoted the elongation of transcription by pol II.

In the second case, HMG-14 was incubated with MCs before the formation of preinitiation transcription complexes with the components in the HeLa whole-cell extract. If HMG-14 facilitates the assembly of preinitiation complexes on the chromatin templates, additional transcripts should have been formed, resulting in an increase in the final level of elongated transcripts. However, after 90 min of incubation with high concentrations of unlabeled nucleotides to extend the initiated RNAs, similar levels of full-length transcripts (1457 nucleotides or longer) were obtained whether or not the MCs were preincubated with exogenous HMG-14. Thus, HMG-14 had no effect on the total number of transcription complexes assembled at the late SV40 MC promoter. At earlier times (5 and 20 min, Fig. 3B), the generation of a higher level of full-length transcripts from the MCs in the presence of recombinant HMG-14 simply reflects a faster rate of elongation, as expected from the results of Fig. 3A. Thus, exogenous HMG-14 did not stimulate the

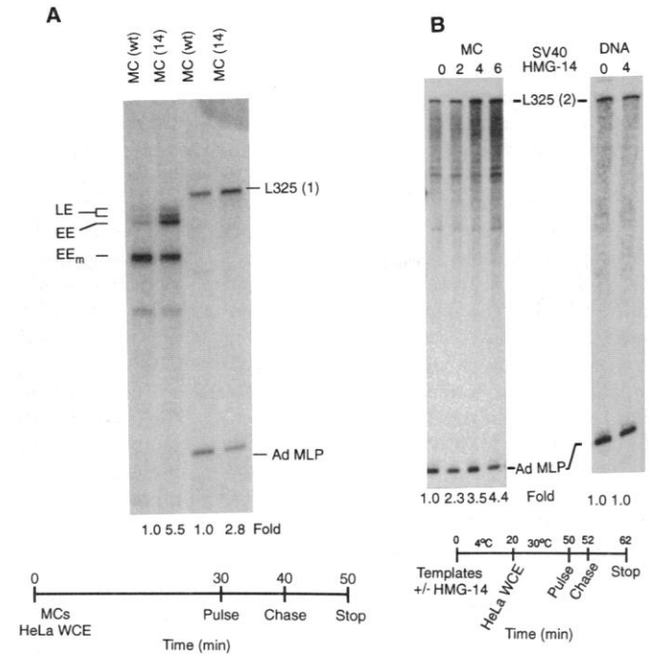

Fig. 2. (**A**) In vitro transcription analysis of MCs isolated from either the parental CV-1 cell line [MC (wt)] or the cell line overexpressing HMG-14 mRNA [MC (14)]. Transcripts from SV40 MC early (EE and LE) and late [L325 (1)] promoters and from internal control promoters (EE_m and Ad MLP) on naked DNA templates are indicated. The fold activation by HMG-14 is indicated in each case at the bottom of the lane (*12*). Each reaction contained SV40 MC (40 ng) (DNA content), a marked SV40-containing plasmid DNA (10 ng) (so that transcripts, that is, EE_m, were distinguishable from viral transcripts upon RNA analysis), pFLBH DNA template containing the adenovirus type 2 major late promoter (20 ng) (Ad MLP) (*25*), and poly[*d*(I-C)]·poly[*d*(I-C)] (300 ng). The in vitro transcription assay and RNA analysis are described in (*12*). The two left lanes, displaying the SV40 early transcripts, were exposed roughly three times longer than the lanes displaying the late transcripts. A time sequence (in minutes) of the protocol is shown at bottom. (**B**) In vitro transcription analysis of SV40 MCs and SV40 viral DNA in the presence or absence of purified, recombinant human HMG-14 (*13*). Transcripts from the SV40 major late promoter [SV40 L325 (2)] and from an internal control promoter (Ad MLP) are indicated. Templates were incubated with either bovine serum albumin (BSA, four molecules per nucleosome; lane labeled 0) or the indicated amounts of recombinant HMG-14 (two, four, or six molecules per nucleosome), followed by in vitro transcription (*12*). A time sequence (in minutes) of the protocol (with temperature conditions) is given at bottom.

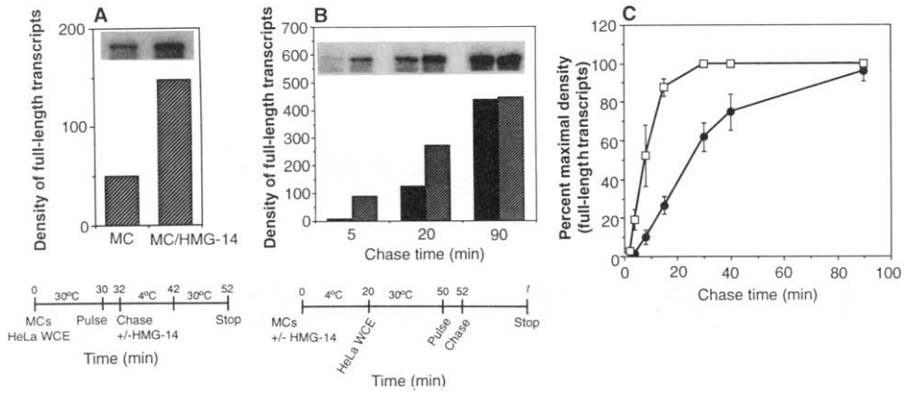

Fig. 3. (**A**) Effect of HMG-14 on elongation. BSA or recombinant HMG-14 (four molecules per nucleosome) was added after the initiation of transcription (*12*). (**Inset**) Autoradiograms of 1457-nucleotide full-length transcripts from the SV40 MC late promoter. Bars represent the relative density of these late transcripts, normalized in each reaction to the internal control Ad MLP transcripts from naked DNA (*14*). A time sequence (in minutes) of the protocol (with temperature conditions) is given at bottom. (**B**) Effect of HMG-14 on initiation. MCs were incubated with either recombinant HMG-14 (four molecules per nucleosome, shaded bars) or BSA (four molecules per nucleosome, solid bars), followed by in vitro transcription. The transcripts were analyzed as in (A). A time sequence (in minutes) of the protocol (with temperature conditions) is given at bottom. (**Inset**) Autoradiograms of 1457-nucleotide full-length transcripts from the SV40 MC late promoter. (**C**) Quantitative analysis of the stimulation by HMG-14 of the rate of elongation. Five separate experiments were performed and analyzed as described in (B). The levels of 1457-nucleotide full-length MC transcripts (open squares and closed circles: with or without exogenous HMG-14, respectively) are plotted versus time after the addition of unlabeled nucleotides (chase). As a comparison of data from several experiments, the levels of MC transcripts are presented as percentage maximal density, that is, the level relative to the maximal plateau level obtained in that experiment from MCs in the presence of recombinant HMG-14.

initiation of transcription on the transcriptionally competent (9) SV40 MCs.

Having established that elongation, not initiation, is affected by HMG-14, we measured the degree to which the rate of elongation is affected by elevated levels of HMG-14. In a manner similar to that of the previous experiments, preinitiation complexes were allowed to form, followed by the initiation of transcripts in the presence of labeled nucleotides for 2 min to label the 5′ terminus of each transcript. The average rate of elongation was then calculated by measuring the generation of transcripts of a certain length with time in the presence of high levels of unlabeled nucleotides. The data from five independent experiments are summarized in Fig. 3C. The time required to achieve half maximal levels of full-length transcripts was 24 min for MCs and 7 min for MCs with added HMG-14, which indicates that there was a 3.5-fold increase in the rate of elongation on MCs upon the addition of HMG-14. The average elongation rates obtained from the data in Fig. 3C were 60 nucleotides per minute for MCs and 210 nucleotides per minute for MCs enriched in HMG-14.

The alteration in chromatin structure from inactive to active templates must involve multiple steps that are potentiated by several cellular factors. Some factors might potentiate the initiation of transcription and others might potentiate the elongation of transcription. In vivo–assembled SV40 MCs, which contain nucleosome-deficient, transcriptionally competent promoters (9), normally associate with non-saturating levels of HMG-14 (Fig. 1B). Whether HMG-14 plays any role in the generation in vivo of these transcriptionally competent promoters must be explored further. However, once these active promoter regions are formed, HMG-14 will not further enhance initiation of transcription (Fig. 3B).

The presence of factors in vivo that facilitate elongation by pol II on chromatin templates has been suggested by the observation that elongation by pol II can be severely inhibited in the presence of reconstituted nucleosomes (*15*), in contrast to the rapid elongation rates observed in vivo (*16*). The ability of HMG-14 to alleviate the inhibitory effect of nucleosomes on elongation by pol II indicates that the association of HMG-14 with nucleosomes is at least part of the process that confers a transcriptionally active structure on chromatin.

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10. Expression of human HMG-14 mRNA in CV-1 cells. Full-length cDNA for human HMG-14 from pSVL14s (*17*) was inserted at the Bam HI site in the mammalian expression vector pLEN (*18*) to construct pLEN14s. CV-1 cells were transfected through the $CaCl_2$ procedure (*19*) with pLEN14s (20 μg) and pCMV-Neo (1 μg) (*20*). The cells were washed twice 16 hours later with serum-free Dulbecco's modified Eagle's medium (DMEM) and grown in DMEM with 10% calf serum. The cells were split 36 hours after transfection and grown in selective medium [DMEM, 10% calf serum, G418 (1 mg/ml)]. Two to three weeks later, G418-resistant cells were harvested and analyzed for the expression of human HMG-14 mRNA.
11. Simian virus 40 MCs were isolated by extraction from nuclei and purified by sucrose gradient centrifugation as described (*8*). To isolate SV40 viral DNA, confluent plates of CV-1 cells were infected with SV40 at 10 plaque-forming units per cell. At 48 hours after infection, the viral DNA was isolated from cells lysed with 0.6% SDS and 1 M CsCl (*21*) and banded twice in CsCl isopycnic gradients.
12. Unless indicated elsewhere, each reaction mixture contained MCs (DNA content of 200 μg) or SV40 viral DNA (200 μg), pFLBH DNA (50 μg), and poly[*d*(I-C)]·poly[*d*(I-C)] (200 μg). All of the in vitro transcription reactions were performed as outlined at the bottom of each figure, with the use of a preincubation pulse-chase protocol with incubations at 30°C (*8, 22*). HeLa whole-cell extract (WCE) (*23*) provided all the necessary transcription factors. Nucleotide concentrations to label the RNAs during the pulse were 2 μM [α-^{32}P]-uridine 5′-triphosphate (UTP) (20 μCi) and 30 μM of the three unlabeled nucleoside triphosphates. Nucleotide concentrations to chase the transcripts into longer products were 1 mM for UTP and 330 μM for the three remaining nucleoside triphosphates. Specifically initiated RNAs were selected by hybridization with single-stranded M13-SV40 and M13-adenovirus recombinant DNA probes and trimmed by ribonuclease T1 digestion. Probe-1 and probe-2 are single-stranded M13-SV40 recombinant DNAs that contain the late template strand of the SV40 genome from positions 4770 to 874 and 294 to 1782, respectively. These probes lead to the generation of T1-resistant transcripts from the major late SV40 promoter (L325) of 549 nucleotides and 1457 nucleotides, respectively. The probe for SV40 early transcripts contains the early template strand of the SV40 genome from positions 874 to 4770, which leads to the generation of T1-resistant transcripts of 468 to 473 and 510 to 512 nucleotides from the EE and LE promoters, respectively. The resulting T1-resistant transcripts were resolved by 8.3 M urea–5% polyacrylamide gel electrophoresis and analyzed by ImageQuant software on a PhosphorImager (Molecular Dynamics). The numbers for the fold activation by HMG-14 were obtained after correcting for the level of transcripts from the internal control promoters (EE_m or Ad MLP). They were then normalized to levels of transcripts from MCs isolated form parental cells or from SV40 viral DNA in the absence of exogenous HMG-14. Thus, the fold activation for MC or viral DNA in the absence of exogenous HMG-14 is 1.0.
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Control of Topographic Retinal Axon Branching by Inhibitory Membrane-Bound Molecules

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Retinotopic map development in nonmammalian vertebrates appears to be controlled by molecules that guide or restrict retinal axons to correct locations in their targets. However, the retinotopic map in the superior colliculus (SC) of the rat is developed instead by a topographic bias in collateral branching and arborization. Temporal retinal axons extending across alternating membranes from the topographically correct rostral SC or the incorrect caudal SC of embryonic rats preferentially branch on rostral membranes. Branching preference is due to an inhibitory phosphatidylinositol-linked molecule in the caudal SC. Thus, position-encoding membrane-bound molecules may establish retinotopic maps in mammals by regulating axon branching, not by directing axon growth.

Most axonal connections in the central nervous system are organized in a precise topographic manner. The projection of retinal ganglion cell axons to the optic tectum in nonmammalian vertebrates, or to the homologous SC in mammals, is widely used to investigate mechanisms that control the development of topographic connections. In a mature projection, the temporal-nasal axis of the retina maps along the rostral-caudal axis of the target. In vivo and in vitro studies support the hypothesis that a position-dependent expression of molecules in the retina and its target, in either graded or restricted distributions, governs the topographic targeting of retinal axons (*1*). For example, in vivo studies in developing frogs, fish, and chicks have shown that retinal axons are guided or restricted to their topographically appropriate part of the tectum (*2*). Compelling in vitro evidence for the action of position-encoding molecules comes from the membrane stripe assay, in which temporal retinal axons extending on alternating lanes of membrane from the rostral and caudal tectum prefer to grow on rostral lanes (*3–5*). This growth preference is due to a repulsive phosphatidylinositol (PI)-anchored molecule preferentially associated with the caudal tectum, which is thought to act in vivo by restricting growing temporal retinal axons to their topographically appropriate positions (*4, 6–8*).

Different mechanisms are emphasized in the development of the rat retinocollicular projection (*9, 10*). At embryonic ages, temporal and nasal retinal axons show no distinction in their topographic targeting across the rostral-caudal SC axis (*9*). However, when tested in the membrane stripe assay at these ages, temporal retinal axons show the same strong preference for rostral membrane lanes that chick and fish axons do (*9*). Taken together, these findings indicate that position-dependent information is present in the embryonic SC, but this information does not guide or restrict the growth of retinal axons in vivo. The first observed indication of topographic specificity in the retinocollicular projection is a bias in the distribution of side branches extending from retinal axons that have already grown past their correct topographic locations—temporal axons preferentially branch in the rostral SC and nasal axons in the caudal SC (*9*). A likely role, then, for

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