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20. We thank R. Dixon for the cDNA-library; T. Shinohara, for providing pBCarrestin (*6*); B. Cullen for a sample of pBC12/CMV/IL-2 (*9*); H. Dohlman, P. Falardeau, P. Schnitzler, W. Lorenz, J. Arrizza, S. Senogles and J. Pitcher for advice; C. Stone, G. Irons, and K. Daniel for technical assistance with the transfections, cell culture, purification of βARK and β_2 receptors, and parts of the sequencing; and D. Addison for secretarial assistance. Supported in part by NIH grants HL16037 and DK19318 and a fellowship (M.J.L.) from the Deutsche Forschungsgemeinschaft.

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An RNA Polymerase II Transcription Factor Shares Functional Properties with *Escherichia coli* σ^{70}

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A mammalian transcription factor, which, along with other factors, is essential for accurate initiation of transcription from promoters by RNA polymerase II, has been found to regulate the interaction of polymerase and DNA. This factor, designated βγ, drastically reduces the affinity of RNA polymerase II for free DNA containing either promoter or nonpromoter sequences. In this respect, βγ functions as does the bacterial transcription initiation factor σ^{70}, which expedites the binding of *Escherichia coli* RNA polymerase to promoters in part by accelerating dissociation of the polymerase from nonpromoter sites in DNA.

INITIATION OF MRNA SYNTHESIS IS A key control point in the expression of many eukaryotic genes. Such diverse signals as heat shock, glucocorticosteroids, and lymphokines regulate the expression of genes by controlling when and how often RNA polymerase II initiates and synthesizes their mRNA. As a consequence, our ultimate understanding of eukaryotic gene regulation relies on the ability to understand, at the molecular level, how RNA polymerase II recognizes, binds to, and initiates transcription from its promoter.

Transcription initiation is an elaborate biochemical process governed by interactions between RNA polymerase II, multiple accessory transcription factors (*1–6*), an adenosine triphosphate (ATP)–deoxyadenosine triphosphate (dATP) [ATP(dATP)] cofactor (*7, 8*), and promoter DNA. Although the mechanism of initiation is poorly understood, a working model has emerged from analyses of partially purified transcription systems. This model proposes that one or more accessory factors bind directly to promoter sequences to form the first stable intermediate in initiation. RNA polymerase II, assisted by additional factors, then recognizes and assembles with this "initial" complex to form a functional preinitiation complex (*2, 5, 9, 10*). In a step requiring an ATP(dATP) cofactor, the preinitiation complex is then converted to an "activated" complex, which is capable of initiating RNA synthesis rapidly after addition of the remaining ribonucleoside triphosphates (*8*).

To define the roles played by the accessory transcription factors in initiation, we have assembled a highly purified, reconstituted RNA polymerase II transcription system from rat liver (*3–6*). Synthesis of accurately initiated transcripts in this system requires, in addition to RNA polymerase II, five accessory factors that comprise two functional classes: (i) "promoter recognition" factors (τ and ϵ), which interact with template DNA to form the initial complex at the promoter (*6, 11*) and (ii) "RNA chain initiation" factors (α, βγ, and δ), which subsequently, along with RNA polymerase II, assemble the functional preinitiation complex. Although no α, βγ, or δ is required for assembly of the initial complex by τ and ϵ, each factor ultimately becomes an integral component of the fully assembled preinitiation complex (*10*).

In *Escherichia coli*, transcription initiation factor σ^{70} regulates the binding of RNA polymerase to promoters. Biochemical studies have established that σ^{70} not only increases the affinity of polymerase for promoter sequences, but also dramatically decreases the affinity of polymerase for nonpromoter sequences (*12*). In the absence of σ^{70}, the bacterial "core" polymerase binds DNA avidly but nonspecifically, with a half-life of approximately 60 min at 25°C. In the presence of σ^{70}, RNA polymerase binds weakly to nonpromoter sequences, but binds to promoter sequences at least 10^4-fold more strongly and with a half-life of nearly 60 hours. Thus, by ensuring that *E. coli* RNA polymerase binds tightly to its promoter but weakly to nonpromoter sites in DNA, σ^{70} markedly accelerates assembly of an active preinitiation complex.

Like the bacterial core polymerase, mammalian RNA polymerase II has been found to bind stably to free DNA (*13*). In the course of our investigations, however, we observed that excess free DNA added after formation of the initial complex by τ and ϵ did not prevent assembly of RNA polymerase II into the functional preinitiation complex in the presence of transcription factors α, βγ, and δ (*5, 14*). This result suggested that one or more of these liver transcription factors might act to prevent formation of nonproductive binary complexes of polymerase and free DNA.

To explore this possibility further, we developed an electrophoretic mobility shift assay, based on the procedure of Fried and Crothers (*15*), to monitor formation of binary complexes of RNA polymerase II and free DNA. As shown in Fig. 1, purified RNA polymerase II (*16*) will bind strongly to a 60-bp DNA fragment containing the

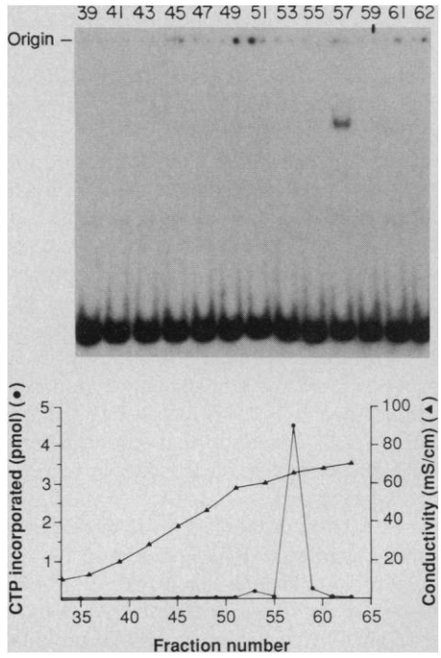

Fig. 1. Cochromatography of RNA synthesis and DNA-binding activities during TSK DEAE-NPR HPLC of RNA polymerase II. TSK DEAE-NPR HPLC was carried out as described (*16*). (**Top**) Binding reactions (*24*) contained 250 ng of Nde I–cut pUC18, 5 ng of a 60-bp [5′-^{32}P]-labeled DNA fragment containing the adenovirus −50 to +10 sequence [Ad(−50 to +10)] (*5*), and 0.2 μl of the indicated fraction. (**Bottom**) RNA synthesis reactions were performed as described (*16*) with heat denatured calf thymus DNA as the template. Conductivity was measured with a Radiometer-Copenhagen CDM-83 conductivity meter.

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adenovirus 2 major late promoter to yield a stable binary complex that can be analyzed by native gel electrophoresis. RNA synthesis and DNA-binding activities cochromatograph on TSK DEAE-NPR (Hewlett-Packard) high-performance liquid chromatography (HPLC) during purification of polymerase, indicating that RNA polymerase II is responsible for binary complex formation. At low ratios of polymerase to DNA, a single electrophoretically shifted species was observed (Fig. 2, lanes 1 to 7); the half-life of this complex is more than 60 min (Fig. 3) (*14*). At higher ratios of polymerase to DNA, nearly all the DNA fragment was bound and two shifted species were observed (Fig. 2, lanes 8 to 10), suggesting that these short DNA fragments can accommodate the binding of more than one molecule of polymerase.

RNA polymerase II exhibits no strong sequence preference in formation of the binary complex. First, RNA polymerase II bound to all DNA fragments tested. These included fragments containing the adenovirus 2 major late promoter (Fig. 2); the mouse interleukin-3 promoter; a mutant spinach chloroplast promoter, trnM2-psbA (*17*) (Fig. 3B); and five different fragments containing portions of the mouse interleukin-2 coding region (*14*). Second, DNA fragments containing both class II promoter and nonpromoter sequences effectively inhibited the binding of polymerase to DNA fragments containing the adenovirus 2 major late promoter (*14*), demonstrating that RNA polymerase II is unable to bind selectively to its promoter in the absence of accessory transcription factors. RNA polymerase II, therefore, like the *E. coli* core polymerase, interacts stably and nonspecifically with free DNA to form a binary complex.

Because we previously observed that free DNA failed to prevent RNA polymerase II from associating with preformed initial complexes in the presence of α, βγ, and δ, even though polymerase could bind DNA in the absence of the accessory transcription factors (*5, 14*), we reasoned that one or more of the liver transcription factors might prevent formation of nonproductive binary complexes by reducing the affinity of polymerase for naked DNA. To test this possibility, the liver factors were assayed for their ability to regulate formation of the binary complex. We observed a substantial decrease in the amount of binary complex formation when purified transcription factor βγ (*5*) was included in binding reactions along with RNA polymerase II and DNA fragments containing the adenovirus 2 major late promoter (Fig. 4, lanes 1 to 4). None of the factors α, δ, ϵ, or τ inhibited binary

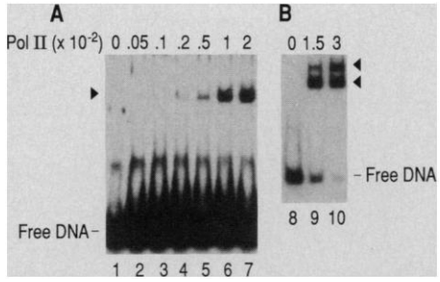

Fig. 2. DNA-binding activity of RNA polymerase II. Binding reactions (*24*) included RNA polymerase II and DNA fragment [5′-^{32}P]Ad(−50 to +10) as indicated in the figure. (**A**) Five nanograms of DNA fragment. (**B**) One nanogram of DNA fragment. The arrowheads indicate the positions of complexes of RNA polymerase II and DNA.

complex formation, although βγ inhibited binary complex formation substantially at ratios of βγ to RNA polymerase II optimal for promoter-specific transcription in the reconstituted liver system. Moreover, βγ inhibited the binding of RNA polymerase II to all other sequences tested, including DNA fragments containing the mouse interleukin-3 promoter, the chloroplast DNA sequence trnM2-psbA, and mouse interleukin-2 coding sequences (*14*). Because βγ alone appeared not to interact with DNA (Fig. 4, lanes 5 to 8), these results strongly suggested that βγ alters the ability of RNA polymerase II to bind naked DNA.

To determine whether βγ is capable of disrupting preformed binary complexes, we performed the following experiment. First, binary complexes were formed by preincubation of RNA polymerase II with radioactively labeled DNA. These complexes were then diluted with an excess of the same, unlabeled DNA, and βγ was added. The rate of complex dissociation was monitored by the electrophoretic mobility shift assay. As shown in Fig. 3, βγ promotes the rapid dissociation of RNA polymerase II from the DNA. In the presence of βγ, the half-life of the binary complex is less than 5 min for all fragments tested. Transcription and binary complex–dissociating activities cochromatograph on TSK SP-5PW HPLC during purification of transcription factor βγ (Fig. 5), indicating that βγ regulates the DNA-binding properties of RNA polymerase II.

Mammalian RNA polymerase II resem-

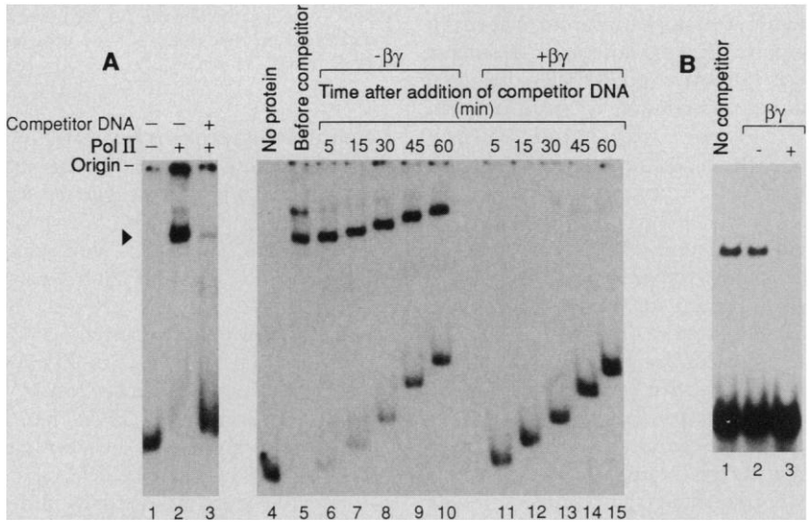

Fig. 3. Dissociation of preformed binary complexes by transcription factor βγ. (**A**) Lanes 1 and 4, free [5′-^{32}P]Ad(−50 to +10); lanes 2 and 3, DNA binding reactions (*24*) contained 1 ng [5′-^{32}P]Ad(−50 to +10) and 0.01 units of RNA polymerase II; lane 3, a 20-fold excess of competitor DNA [20 ng of unlabeled Ad(−50 to +10)] was added to reaction mixtures before addition of RNA polymerase II; lanes 5 to 15, binary complexes were formed in 160 μl of reaction mixture as described (*24*), with 8 ng of [5′-^{32}P]Ad(−50 to +10) and 0.08 units of RNA polymerase II. After a 20-min incubation, 10 μl of the reaction mixture was removed and loaded onto a 4% polyacrylamide gel prepared as described (*24*). To the remainder was added a 20-fold excess of unlabeled competitor DNA [150 ng Ad(−50 to +10)]. The reaction mixture was immediately divided into two aliquots, and 40 ng of βγ (*25*) was added to one aliquot. At the times indicated, 10 μl was removed from each aliquot and loaded onto the gel. The arrowhead indicates the position of the RNA polymerase II–specific binary complex (lanes 1 to 3). In lanes 4 to 15, the extent of migration varies because samples were loaded into each lane of the gel at different times. (**B**) Binary complexes were formed in 35 μl of reaction mixture as described (*24*) with 2 ng of a 50-bp [5′-^{32}P]-labeled DNA fragment containing the trnM2-psbA sequence (*17*) and 0.01 units of RNA polymerase II. After a 20-min incubation, 10 μl of the reaction mixture (lane 1) was removed and loaded onto a polyacrylamide gel prepared as described (*24*). To the remainder was added a 20-fold excess of unlabeled trnM2-psbA fragment. The reaction mixture was divided into two aliquots, and 10 ng of βγ (*25*) was added to one aliquot. After 5 min, 10 μl of each aliquot was loaded onto the gel (lanes 2 and 3).

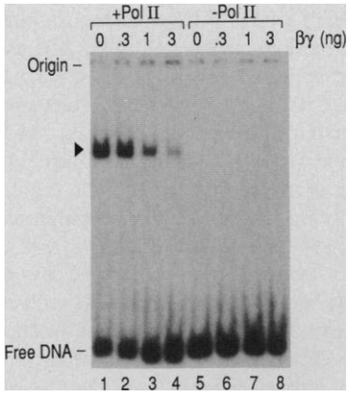

Fig. 4. Transcription factor βγ alters the ability of RNA polymerase II to bind DNA. Binding reactions (*24*) contained 0.3 ng of [5′-^{32}P]Ad(−50 to +10) and the indicated amount of transcription factor βγ, fraction V (*5*), with (lanes 1 to 4) or without (lanes 5 to 8) 0.005 units of RNA polymerase II. The arrowhead indicates the position of the RNA polymerase II–specific binary complex.

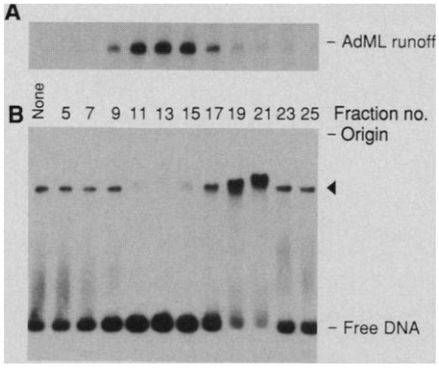

Fig. 5. Transcription activity and binary complex dissociating activity cochromatograph during TSK SP-5PW HPLC of βγ. (**A**) Assays for transcription factor βγ were performed as described (*5*) with 0.5 μl of the indicated fraction; reaction mixtures contained 0.1 μg of Nde I–cut pDN-AdML (*8*), which includes nucleotides −50 to +10 of the adenovirus major late promoter inserted into the polylinker of pUC-18, as template. AdML runoff, 260-nucleotide runoff transcript from the adenovirus 2 major late promoter on pDN-AdML. (**B**) Binary complexes were formed as described in the legend to Fig. 3A. After addition of a 20-fold excess of unlabeled competitor DNA, the reaction mixture was divided into 10-μl aliquots, and 0.5 μl of the indicated fraction was added. Reactions were incubated for a further 10 min and analyzed by polyacrylamide gel electrophoresis. Electrophoresis was performed as described (*24*) except that 5% (v/v) glycerol was included in the gel. The arrowhead indicates the position of the RNA polymerase II–specific binary complex.

bles the *E. coli* core polymerase, in that both enzymes bind tightly and nonspecifically to free DNA; yet, in order to initiate mRNA synthesis correctly, each must be capable of locating promoter sequences amid an excess of nonpromoter chromosomal DNA. In *E. coli*, a single accessory transcription factor, σ^{70}, performs multiple functions that enable RNA polymerase to locate its promoter, establish an active preinitiation complex, and initiate transcription. σ^{70} transforms the *E. coli* core polymerase into a sequence-specific DNA-binding protein by increasing its affinity for promoter sequences and by suppressing its binding to nonpromoter sequences. In mammalian cells, at least some of the functions provided by σ^{70} in bacterial gene transcription appear to be carried out by individual factors during promoter-specific transcription by RNA polymerase II. We have shown that purified transcription factor βγ, a factor essential for accurate initiation in the reconstituted liver transcription system, could carry out one such function by suppressing the binding of RNA polymerase II to free DNA.

The ability of βγ to regulate the binding of RNA polymerase II to free DNA may be an important feature of its function in promoter-specific transcription initiation. Our recent findings suggest that α, βγ, and perhaps δ are required for correct binding of RNA polymerase II to initial complexes at the promoter (*6, 10*). Thus, one of the functions of βγ could be to enhance the specificity with which RNA polymerase II recognizes and binds to the preinitiation complex, in part by preventing the nonproductive association of polymerase with free DNA.

The precise relation between βγ and other mammalian transcription factors is presently unclear. Because of their similar subunit compositions, it has been suggested that βγ is analogous to RAP30/74 (*18*), which has been shown to be identical with transcription factor IIF (TFIIF) (*19*). RNA polymerase–associated protein (RAP) 30/74 (TFIIF), however, is reported to have a native molecular mass of 70 to 90 kD (*20*), whereas βγ is approximately 250 kD (*5*). More significant, RAP 30/74 has been shown to possess DNA helicase activity (*21*), whereas βγ exhibits no detectable helicase activity when measured by the same methods (*22*).

Note added in proof: Recently, it has been reported that the native molecular mass of TFIIF, determined by gel filtration, is 220 kD (*23*).

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16. RNA polymerase II was purified from the rat liver nuclear extract [fraction I, in (*4*)] by ammonium sulfate fractionation, followed by chromatography on successive phosphocellulose and DEAE-cellulose columns performed as described (*3*). Active fractions from DEAE-cellulose were concentrated by precipitation with 0.35 g of $(NH_4)_2SO_4$ per milliliter. The pellet was dissolved in 200 μl of buffer F [40 mM tris-HCl (pH 7.5), 0.5 mM EDTA, 1 mM dithiothreitol (DTT), and 10% (v/v) glycerol] and applied to a 7.5 mm by 600 mm 4000 SW Spherogel TSK HPLC column (Beckman) equilibrated with buffer F containing 0.5 M KCl. The column was eluted at 0.5 ml/min, and 0.5-ml fractions were collected. Active fractions were pooled and purified further by TSK DEAE-NPR HPLC on a 4.6 mm by 33 mm column (Hewlett-Packard) eluted at 0.6 ml/min with a 9-ml gradient from 0.1 to 0.5 M KCl in buffer C [40 mM tris-HCl (pH 7.9), 0.5 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol]. As judged by SDS–polyacrylamide gel electrophoresis, RNA polymerase II, which was assayed essentially as described [H. G. Hodo and S. P. Blatti, *Biochemistry* **16**, 2334 (1977)], was more than 95% from IIA [M. E. Dahmus, *J. Biol. Chem.* **258**, 3956 (1983)]. The specific activity of purified rat liver RNA polymerase II was ~500 units/mg (1 unit = 0.25 nmol of cytidine triphosphate incorporated in 10 min at 37°C).
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24. Standard binding reactions contained 20 mM Hepes-NaOH (pH 7.9), 20 mM tris-HCl (pH 7.9), 50 mM KCl, 2 mM DTT, 7% (v/v) glycerol, bovine serum albumin at 0.5 mg/ml, 2% (w/v) polyvinyl alcohol, and DNA and protein fractions as indicated in the figure legends. Unless otherwise indicated, reactions were incubated for 20 min at 28°C and then separated by electrophoresis at 75 V for 2.5 to 3 hours in a polyacrylamide gel containing 4% acrylamide, 0.1% bisacrylamide, 89 mM tris, 89 mM borate, and 2 mM EDTA.
25. Transcription factor βγ (~20 μg/ml) used in the experiments shown in Figs. 3 and 5 was purified from the livers of 250 rats as described (*5*), except that the acetone fractionation step was omitted, and DEAE chromatography was carried out using a semipreparative (21.5 mm by 150 mm) TSK DEAE-5-PW HPLC column (Beckman).
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A Major Direct GABAergic Pathway from Zona Incerta to Neocortex

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Retrograde fluorescent tracers were used to demonstrate a previously unknown but sizable direct γ-aminobutyric acid (GABA)–containing neuronal pathway from the zona incerta to the neocortex in rats. This incertocortical pathway was found to project bilaterally to the entire neocortex and exhibited a rough corticotopic organization. Many of the zona incerta neurons projecting to the parietal and occipital cortices could also be immunohistochemically stained with antibodies to glutamic acid decarboxylase and GABA. Few of these neurons were immunoreactive to tyrosine hydroxylase antibodies, which identify dopamine-containing neurons. Injections in the frontal and entorhinal cortices labeled many neurons near or within the dopaminergic A13 subdivision of the zona incerta. In addition, the incertocortical system was found to be significantly larger during early postnatal (2 to 3 weeks) development. The projection pattern of this newly discovered pathway resembles that of the monoaminergic and cholinergic systems, arising from the brainstem and forebrain, suggesting possible similarities of function.

THE DORSAL THALAMUS OF THE DIencephalon was once considered to be the exclusive source of afferents to the neocortex. However, a number of direct nondiencephalic neocortical afferent systems have been described during the past two decades. These direct ascending systems include the monoaminergic inputs from the locus ceruleus, the raphe nuclei, and ventral tegmental area in the brainstem (*1*), and the cholinergic inputs from the basal forebrain area (*2*). One of the unique features of these transmitter-specific systems is their more diffuse and bilateral projection pattern to all cortical areas as compared to the dorsal thalamic nuclei, which have very specific and ipsilateral connections to different neocortical areas (*3*). These ascending systems are also known to be closely related to early cortical development and plasticity (*4*).

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Although electrical stimulation in the general region of the ventral thalamus produces arousal and cortical desynchronization (*5*), no study has yet demonstrated direct neocortical projections from any region of the ventral thalamus [such as zona incerta (ZI)] (*6*).

We report here the existence of widespread projections from the ZI to the entire neocortex. These projections were discovered through the use of recently developed, very sensitive fluorescent retrograde tracers. Furthermore, in experiments that combined the retrograde tracing with immunohistochemical approaches, many of these incertocortical projecting neurons were found to stain positively for glutamic acid decarboxylase (GAD) or γ-aminobutyric acid (GABA).

Our conclusions are based on a total of 60 rats injected with fluorescent tracers either unilaterally or bilaterally into several cortical areas. The cortical projection pattern of ZI neurons was determined both in adult (n = 40) and young (2 weeks, n = 10; 3 weeks, n = 10) rats. Small injections (0.5 to 1 μl) of either rhodamine-coated microspheres (RCMs) or fluorescein-coated microspheres (FCMs) or Fluoro-Gold were stereotaxically placed into the frontal (4 cases), motor (8 cases), somatosensory (45 cases), auditory (4 cases), entorhinal (4 cases), and visual (15 cases) cortices. After survival times of from 4 to 10 days, the rats were perfused with either 10% formalin or 3.0% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. Brain sections (30 to 100 μm thick) were cut with a vibratome. Antibodies to the synthetic enzyme GAD and GABA were used as specific markers for GABAergic neurons. Antiserum to tyrosine hydroxylase (TH) was used as a marker for dopamine-containing neurons. Standard immunohistochemical methods, including fluorescence and the modified ABC technique, were used for the identification of neurons containing dopamine and GAD or GABA (*7*). Combined retrograde fluorescent tracers and immunohistochemical methods were also used on the same sections to elucidate the specific transmitter that might be contained in the incertocortical projections (*8*). An epifluorescent microscope (Nikon) was used to visualize the precise location of neurons with fluorescent retrograde tracers.

Whereas the thalamocortical connections revealed in these studies were consistent with those previously reported in the literature, the presence of large numbers of retrogradely labeled neurons in the ZI was unexpected. For example, after dyes were injected into the primary somatosensory cortex (SI), retrogradely labeled neurons were found in the ventroposterior nucleus (VP), the posterior nuclear complex (PO), the centrolateral nucleus (CL), the posterior division of the hypothalamic nuclei (HP), (Fig. 1, A to D), and the ventromedial thalamic nucleus (VM). However, the most intriguing finding was that many cells in the dorsolateral region of ZI were labeled (Fig. 1, B and C). In addition, a few scattered labeled neurons were found in the contralateral ZI and bilaterally in the posterior region of the hypothalamus [as had been previously reported (*9*)]. Injections of tracers into the primary visual cortex (VI) produced fewer retrogradely labeled neurons in ZI than after SI injections, and these were primarily located in the ventrolateral subregions of the ZI, and the hypothalamus (Fig. 1, D to F). Although injections of different colored retrograde tracers placed in the SI and VI cortices yielded two separable clusters of labeled cells in the ZI, a significant overlap between these clusters was observed. Moreover, several double-labeled neurons were identified in this overlap zone. After injections into the entorhinal-temporal cortical areas, labeled neurons were found forming a dense cluster, centered medially around the