1993

Analysis of Novel Genes Whose mRNAs Are Enriched in the HVC-Associated Telencephalon of Songbirds

Julia Melinda George

Analysis of Novel Genes Whose mRNAs Are Enriched in the HVC-Associated Telencephalon of Songbirds

A thesis submitted to the Faculty of The Rockefeller University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

by

Julia Melinda George

April 1, 1993

ACKNOWLEDGEMENTS

I am deeply grateful to my thesis adviser, David Clayton, for his enthusiastic support at every stage of this work, for his particular insights at critical moments, and most of all, for his friendship.

I extend my sincere thanks to Fernando Nottebohm, who has always shown extreme generosity with his time and lab resources (particularly his birds).

Hui Jin has collaborated directly in the studies of developmental gene regulation which are described here. Wendy Woods and Maria Huecas provided excellent technical assistance.

Thank you to fellow students Claudio Mello and Kent Nastiuk, for many useful discussions and for frequent technical advice.

Much appreciation also to Barbara O'Loughlin, lab manager extraordinaire, who made the lab environment navigable for me and everyone else in the Nottebohm Lab.

And thanks finally to everyone in the Nottebohm Lab, who contributed to a very pleasant and stimulating scientific environment during my years in New York, and to the members of David Clayton's Lab at the University of Illinois, who continue to offer a fresh perspective on this work in addition to being a very enjoyable group of people.

CONTENTS

INDEX OF FIGURES

INDEX OF TABLES

ABSTRACT

7 cloned cDNAs were selected for analysis due to the enrichment of their corresponding RNAs in the canary HAT, a telencephalic region immediately surrounding and including song nucleus HVC. The goal of the analysis was to determine what kind of protein is encoded by each RNA, whether the RNA distinguishes sites of defined neural plasticity (i.e., the telencephalic song control nuclei or the neurogenic ventricular zone), and whether the RNA's abundance changes at times when anatomical and functional changes are known to occur in these regions. Sequence analysis demonstrated that 4 of these clones encode novel proteins with suggestive or recognizeable functional domains: HAT-2 contains regions of homology to a regulatory domain in Protein Kinase C (PKC) and to a GTPase activating domain in the *bcr* oncogene; HAT-5 encodes a protein kinase; HAT-14 contains a site present in the neural growth-associated protein GAP43 responsible for PKC-regulated calmodulin binding; and HAT-3 contains an 11 amino acid repeated sequence suggestive of a function in membrane recognition or stabilization. Of these, all but HAT-5 are brain-specific, all are expressed fairly widely throughout the telencephalon (as determined by in situ hybridization), and all show differential regulation in at least one of the telencephalic song control nuclei. Expression of HAT-3 RNA appears to increase in several song control nuclei (especially L-MAN) at times when the song circuit is first forming, and antibodies generated against the predicted HAT-3 peptide sequence suggest the protein is localized in

neuritic processes and synapses. These results show the song nuclei are distinguished from surrounding brain regions by their patterns of gene expression, and suggest that complex signal transduction mechanisms are characteristic of the telencephalon.

Chapter 1. Introduction

There are an estimated 30,000 mRNAs expressed in the vertebrate brain, approximately twice as many RNAs as any other tissue (Chikaraishi, 1979). Over half of these are probably exclusive to the brain (Milner and Sutcliffe, 1983a). Gene expression which is unique to the brain is likely to subserve cellular functions which are likewise unique to the brain, such as neurogenesis, neuronal migration, elaboration of neuronal morphology, establishment of specific connections, and long-term modulation of function. But the vast majority of genes expressed specifically or differentially in the brain are completely uncharacterized.

In this thesis, I describe the characterization of 4 novel RNAs that were discovered by study of the songbird brain, and which have definable relatives in the mammalian nervous system as well. Each of the molecules is interesting in its own right, insofar as it may contribute to specific neural processes and functions. Valuable clues to the functions of the individual molecules can be gained by observing their patterns of expression within the songbird brain; in turn, these molecular probes provide new insight into the neurobiology of the song system. Consideration of the molecules as a group, representing a small sample of brain-enriched or brain-specific molecules, is also revealing as it suggests general properties of the population of genes expressed in the brain.

This introductory chapter will briefly address the relative merits of various methods for analyzing gene expression in the brain. Then features which distinguish the songbird as a model for the study of complex neural processes will be discussed. Finally, the preliminary experiments that serve as a starting point for the work described in this thesis will be summarized.

Various methods have been applied to the identification and characterization of genes expressed in the brain

Early studies of brain-specific molecules employed protein fractionation to identify specific candidates. In the first reported screen for brain-specific proteins, Moore identified 5 candidate spots through comparison of 2-dimensional electrophoretic maps of protein extracts from brain and liver (Moore and McGregor, 1965a). One of these, called S-100, was the first soluble brain protein to be biochemically purified (Moore, 1965b). Antisera against the purified protein localized it primarily to glia (Hyden and McEwen, 1966a), and more recent studies indicate it is actually a member of a large family of Ca^{++}-binding modulatory proteins, many of which are not brain-enriched, involved in regulating cell cycle progression, cellular differentiation, and cytoskeletal-membrane interactions (Kligman and Hilt, 1988). Antisera to a second brain-specific protein, also identified in the above screen and designated 14-3-2, were used to purify a protein from rat brain (Marangos, et al., 1975), which was subsequently localized specifically to neurons (Pickel, et al., 1976).

Discovery of enolase activity associated with this protein led to its redesignation as neuron-specific enolase (Bock and Dissing, 1975).

While direct protein fractionation was clearly effective in identifying the above brain-specific molecules, the approach was limited to detecting the few most abundant proteins, those present at a concentration of ~10^{-2} as a fraction of total protein mass. Protein fractionation was subsequently improved through better detection methods and enhanced electrophoretic separation, e.g., resolution of radiolabelled proteins in a 2-dimensional electrophoretic system combining isoelectric focusing and SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). 2-D gel fractionation has been used successfully to identify a variety of proteins that are differentially regulated in nervous tissue, ranging from Aplysia (Barzilai, et al., 1989) to the mammalian cortex (Geschwind and Hockfield, 1989). However, further characterization of interesting spots on a gel remains a considerable challenge, even with the advent of improved microsequencing techniques (Kennedy, et al., 1988).

The application of hybridoma technology to the study of brain-specific proteins introduced a higher level of sensitivity and led directly to reagents which are useful in characterization of the newly identified proteins (McKay and Hockfield, 1982), (Hockfield and McKay, 1983), (Hendry, et al., 1984), (Levitt, 1984). Hybridomas are generated which secrete antibodies to a complex mixture of proteins, and individual cell lines are cloned and screened for specific immunoreactivity. Monoclonal antibodies thus generated can be used directly for localization, quantitation, and purification of their

corresponding antigens, and (when the target is a protein) to generate DNA clones for complete sequence analysis.

However, this approach also has its limitations. Different proteins vary significantly in their antigenicity, so antibodies generated against a complex mixture of proteins may not faithfully represent the population of proteins. An antibody cannot easily be used to quantitate its corresponding antigen in tissue sections due to variability in antibody/antigen interactions. Finally, considerable work is required before significant sequence information is generated.

The work in this thesis is based on a powerful alternative to the protein or antibody-based methods: nucleic acid-based cloning and hybridization techniques are used to measure the concentrations and relative distributions of specific mRNAs, leading directly to the cloning of differentially regulated genes. Numerous investigators have used differential and subtractive hybridization techniques, followed by *in situ* localization and sequence analysis, to identify specific genes expressed in the brain (Branks and Wilson, 1986), (Anderson and Axel, 1985), (Clayton, et al., 1988), (Porteus, et al., 1992). *Differential (or plus/minus) hybridization* involves screening a cDNA library with a probe representing mRNA from the tissue of interest ("plus"), and comparing the hybridization pattern with that obtained with a probe representing a control tissue ("minus"). Clones which give a *differential* signal with the two probes are selected for further analysis. In *subtractive hybridization*, sequences specific to a tissue of interest are enriched by "subtracting out" sequences present in a control tissue. This is accomplished by solution hybridization of

single-stranded probes representing the two tissues. Common sequences will hybridize to form double-stranded cDNA, while "unique" sequences will not. Hydroxyapatite chromatography is used to separate single- and double-stranded cDNA, and the single-stranded cDNA is used to identify corresponding "unique" sequences in a cDNA library.

Nucleic acid-based methods have several advantages compared to protein-based strategies for screening and preliminary analysis. Because reverse transcription of cDNA from RNA does not depend significantly upon the RNA sequence, the corresponding cDNAs are quite representative of the original mRNA pool (Milner and Sutcliffe, 1983a). Also, nucleic acid probes can reliably be used to quantitate RNA in both tissue extracts and tissue sections (Clayton, et al., 1988). The acquisition of DNA sequence data has become a trivial matter, and extensive databases of sequence information exist which contribute to the interpretation of such data. Finally, when antibody probes are desired, they can be easily generated by immunization with short synthetic peptides corresponding to protein sequence encoded by cDNA (Sutcliffe, et al., 1983b).

The latest extension of this general strategy for identifying genes expressed in the brain has been to obtain short sequence "tags" from randomly selected brain cDNA clones (Adams, et al., 1991). With this approach it may be possible to "identify" virtually every gene expressed in the brain, but the critical and labor-intensive process of analyzing the structure, regulation, and function of these molecules remains ahead. If insight is to be gained into the significance and function of the many genes expressed in the brain,

appropriate biological models are needed, in addition to methods for purifying and characterizing macromolecules. The next section discusses the particular features and advantages of studying songbirds, as a vehicle for identifying and analyzing genes of functional significance to brain development and plasticity.

The songbird is a powerful system for identification and characterization of novel gene products

Oscine songbirds (suborder Passeres, order Passeriformes), including the canary and zebra finch, manifest a number of interesting neural functions which are amenable to study (for recent reviews, see (Konishi, 1989), (Bottjer and Johnson, 1992), (Arnold, 1992); these include:

> the development of a learned behavior (song production)
> the development of specific brain regions and interconnections involved in song control
> "neural plasticity" correlated with behavioral change and influenced by sex hormones
> continued production of neurons in adulthood.

A powerful feature of this system is that, simply by analyzing patterns of gene expression in the context of song circuit anatomy, development, and plasticity, correlations may become apparent which suggest functional hypotheses, and further experiments may

become possible to test these hypotheses. Further description of relevant features of this system is presented below.

1) Song learning:

The ability to learn song distinguishes oscine songbirds, including the canary and zebra finch, from non-oscine species of the same taxonomical order, Passeriformes (Kroodsma and Baylis, 1982). In canaries, song learning fluctuates in seasonal cycles. High levels of very stereotyped song are produced in Spring, as part of male courting behavior and territorial defense. In Fall, when mating behavior declines, song is quite variable and learning of new song elements can occur(Nottebohm, 1981a). Zebra finches, in contrast, learn their song during a critical period in development beginning at about 30 days of age and ending by 90 days (Immelmann, 1969). The song of adult birds does not change.

2) The song circuit:

The circuit underlying song learning and production in oscines has been localized to discrete, interconnected regions in the brain, the so-called song control nuclei (Nottebohm, et al., 1976a), (McCasland, 1987). This circuit seems to have evolved to subserve song learning, as it is absent from non-oscine members of the order Passeriformes (Ball, 1990), (Brenowitz, 1991), which do not learn their vocalizations. Major telencephalic elements of the song circuit which comprise the primary focus of this work are indicated

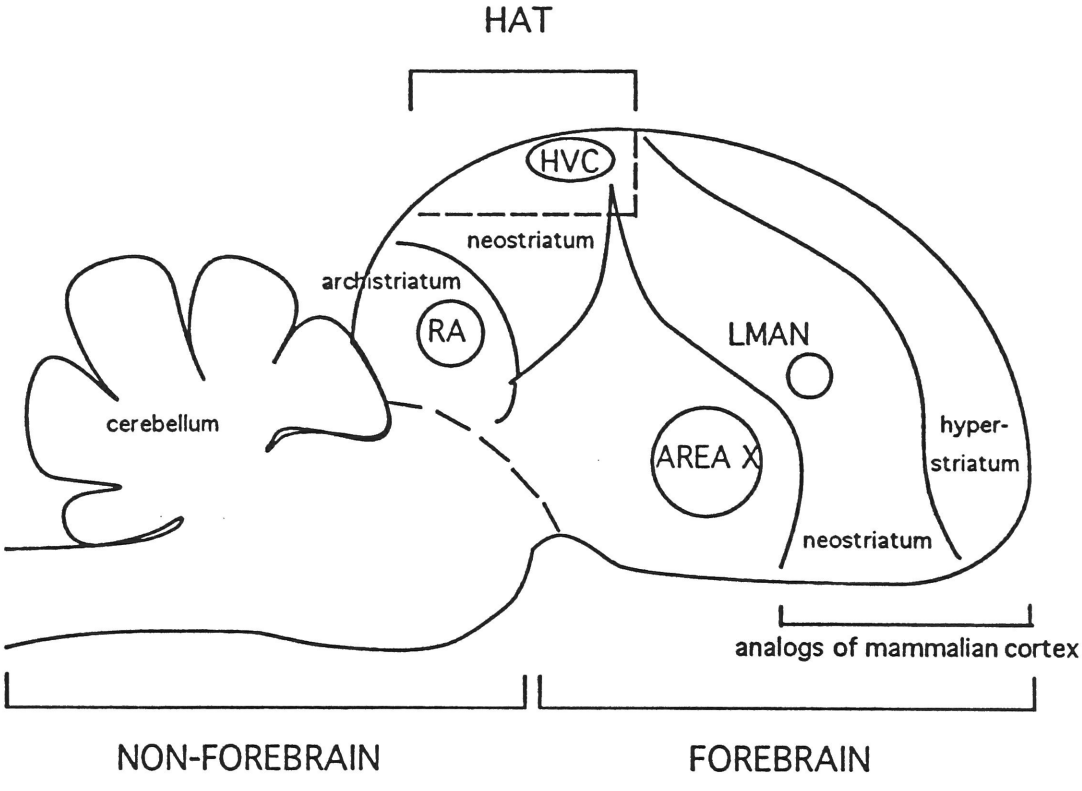
schematically in Figure 1. These include the high vocal center (HVC), the robust nucleus of the archistriatum (RA), the lateral magnocellular nucleus of the anterior neostriatum (LMAN), and Area X of lobus parolfactorius. Also discussed below, but not pictured here, are two nuclei in lower regions of the brain, the dorsolateral nucleus of the anterial thalamus (DLM) and the tracheosyringeal portion of the hypoglossal nucleus (nXIIts), which innervates the muscles of the syrinx. (Other regions, including the interfactial nucleus of the neostriatum (NIF), the uvaeform nucleus of the thalamus (UVA), and the caudal medial neostriatum (NCM), have been recently demonstrated to function in song biology (Nottebohm, et al., 1982), (Konishi, 1989), (Mello et al., 1992). However, these regions have not yet been clearly linked to song learning or to the steroid regulated changes in morphology associated with song acquisition, and they will not be considered further here.)

The principal motor output of this system is the projection from HVC to RA to nXIIts; a so-called "accessory loop" has also been described, comprised of projections from HVC sequentially to Area X, DLM, LMAN, and RA (Nottebohm, et al., 1976a), (McCasland, 1987), (Bottjer, et al., 1989). This latter pathway has been implicated in song learning rather than song production: lesions of these nuclei before closure of the critical period cause disruptions in song, while similar lesions in adult animals have no effect on song (Bottjer, et al., 1984), (Scharff and Nottebohm, 1991).)

Development of specific connections:

Interconnections among song nuclei are developmentally regulated. Two of these connections have been well-characterized in

FIGURE 1. Schematic diagram of the songbird brain. This sagittal view shows the 4 forebrain song control nuclei: high vocal center (HVC), robust nucleus of the archistriatum (RA), lateral magnocellular nucleus of the anterior neostriatum (LMAN), and area X of lobus parolfactorius. The HAT (HVC-associated telencephalon) and non-forebrain dissections are indicated by dotted lines.


HAT
HVC
neostriatum
archistriatum
RA
LMAN
cerebellum
AREA X
hyper-
striatum
neostriatum
analogs of mammalian cortex
NON-FOREBRAIN
FOREBRAIN

zebra finches with respect to timing and pharmacology. A projection from LMAN to RA occurs between days 15 and 25 in young zebra finches (Mooney, 1992). In contrast, the HVC to RA projection is made at 30-35 days in male zebra finches, and is never completed in females of this species (Konishi and Akutagawa, 1985a). LMAN and HVC projections make pharmacologically distinct synapses onto the same RA neurons: the LMAN inputs are sensitive to NMDA antagonism, while the HVC projection is sensitive to a non-NMDA glutamate receptor antagonist (Mooney, 1992). Also, the input from DLM to LMAN is present as early as 15 days, and the projection doubles between 20 and 35 days; the arborization of DLM axons in LMAN seems to rearrange substantially between 35 days and adulthood (Bottjer and Johnson, 1992).

4) Seasonal and developmental changes in song nucleus morphology:

Growth of the song nuclei is correlated with the development of song behavior. Differences in the sizes of particular song nuclei exist among males of these species (which normally sing) and females (which normally do not). In the male canary, HVC and RA volumes (as defined by Nissl staining) are larger in Spring (when song is being produced) than Fall by 50% and 56%, respectively (Nottebohm, 1981a); this is attributed to increases in both cell soma size and dendritic length (DeVoogd and Nottebohm, 1981).

In male zebra finches, there is an increase in the size of HVC and Area X which is attributed to the recruitment of new neurons generated after day 20 (Nordeen, 1988a). Over half of the new neurons in HVC eventually project to RA (Nordeen, 1988b). This

increase in the HVC to RA projection is likewise correlated with an increase in the size of RA (Konishi, 1985a). There is a corresponding decline in the size of LMAN between days 20 and 53, attributable to the loss of approximately half of its neurons (Bottjer, 1985).

5) Gonadal steroid regulation in the song circuit:

Androgen receptors are found in HVC, RA, and LMAN of the telencephalon of canaries and zebra finches (Arnold, 1980a),(Balthazart et al., 1992), while estrogen receptors have been localized to HVC (Gahr et al., 1993). Administration of testosterone to gonadectomized female canaries causes increases in the volumes of HVC and RA of 90% and 53%, respectively (Nottebohm, 1980). Testosterone normally has no effect on the song nuclei of adult female zebra finches (Arnold, 1980b); however, systemic estrogen treatment during early development masculinizes the song circuit, leading to male-like growth of the song nuclei and the development of singing behavior (Simpson and Vicario, 1991a), (Simpson and Vicario, 1991b).

6) Neurogenesis:

Songbirds (and other birds) continue to produce new neurons in adulthood (Goldman and Nottebohm, 1983), from precursor cells in the ependymal lining of the forebrain ventricles. Young neurons migrate outward to sites throughout the forebrain (Alvarez-Buylla and Nottebohm, 1988b) including song nuclei HVC and Area X but not RA and LMAN (Nordeen and Nordeen, 1988a), where they differentiate and become functionally incorporated into previously

established circuits (Paton and Nottebohm, 1984). The functional significance of this is yet unknown, but this process continues to be the focus of much current research interest, due to the relative lack of neurogenesis and repair mechanisms in the adult human brain.

cDNA cloning techniques have been used to explore gene expression in the forebrain of songbirds

Initial efforts to apply cDNA cloning to the songbird system focused on a set of 7 forebrain enriched RNAs (Clayton, et al., 1988), identified by a combination of differential and subtractive hybridization methods. Each of the RNAs was found to have a unique distribution within subsets of cells within the brain, but there was no differential regulation associated with the song circuit or other sites associated with plasticity. Sequence analysis of these clones was also mostly uninformative, due to the relatively small size of the cDNA inserts. In a related study, the population of RNAs expressed in the forebrain was characterized by solution hybridization (Clayton and Huecas, 1990). It was found that at least 1000 genes are enriched in the forebrain relative lower brain regions.

As a consequence of these initial results, the basic screening strategy was modified and refined in several ways (D. Clayton, unpublished). First, greater anatomical focus was achieved by beginning with RNA extracted from the adult male canary HAT (HVC-associated telencephalon), which includes song nucleus HVC and surrounding neo- and hyperstriatum (Figure 1). Second the λgt10 cloning vector was used, yielding lower levels of background

hybridization than the plasmid vector used in the previous experiment (Clayton, et al., 1988) and thus allowing for detection of RNAs expressed at lower levels in the tissue by simple differential (or plus/minus) hybridization screening methods. Third, the library was constructed using the method of Gubler and Hoffman (Gubler and Hoffman, 1983), which resulted in a larger average cDNA insert size. 300 clones from this library were screened in quadruplicate with cDNA probes representing either HAT or non-forebrain (NF), and 16 clones hybridized more strongly to the HAT probe than to the NF probe. Upon further analysis, 7 clones were demonstrated to represent RNA transcripts in songbird forebrain which are enriched in the HAT relative to the NF. These data (unpublished) are summarized in Table I.

TABLE 1. General characteristics of HAT-enriched cloned RNAs selected for analysis

clone	insert size	mRNA [a] size	abundance [b] in HAT	abundance [c] ratio HAT:NF
HAT-1	2400	ND	10^{-5}-10^{-4}	≥3
HAT-2	1696	2200	$5x10^{-4}$	8
HAT-3	1048	1500	$2x10^{-4}$	≥4
HAT-5	1977	2700	10^{-5}-10^{-4}	≥3
HAT-9	538	1800	$1\text{-}2x10^{-3}$	6
HAT-14	2341	1000	$3x10^{-4}$	≥8
HAT-16	2725	3500	10^{-5}-10^{-4}	≥3

[a] Size in bases, as estimated from Northern blots (D. Clayton, unpublished observations)
[b] fractional abundance of each mRNA relative to total mRNA mass, determined by quantitative filter hybridization (D. Clayton, unpublished observations)
[c] ratio of mRNA abundance in the HAT relative to non-forebrain (D. Clayton, unpublished observations)

Aims of this thesis

The 7 HAT clones, described in Table 1, represent a sampling of brain genes which are generally enriched in higher regions of the brain and may be expressed in the song control circuit. The aim of the work presented in this thesis has been to characterize the RNAs and proteins represented by these clones in order to answer the following questions:

1) What does sequence analysis reveal about the type of protein it encodes?
2) In the event the cDNA predicts a novel protein, is that protein actually produced? Where within the cell is the protein localized?
3) Are these HAT-enriched RNAs in fact brain-specific? Are they expressed in neurons?
4) Are these RNAs differentially regulated in the song control system? Are they associated with known sites or periods of plasticity?

Preliminary studies

In my initial work, the 7 HAT cDNAs were completely sequenced and their corresponding RNAs detected in canary brain sections by *in situ* hybridization. Two of the mRNAs, HAT-1 and HAT-16, were difficult to detect in tissues, consistent with their low abundance (see Table 1). Their nucleic acid sequences were unrevealing, suggesting no definitive open reading frames or

homologies to known sequences. For these reasons, HAT-1 and HAT-16 were not analyzed further. A third cDNA, HAT-9, will not be described here, although it is rather strikingly regulated in the songbird forebrain, and its *in situ* hybridization pattern is shown as a control in Chapter 6. The sequence of the relatively small HAT-9 insert (538 bp) has not been informative, and efforts are ongoing to obtain more complete sequence data. The remaining HAT cDNAs, HAT-2, -3, -5, and -14, give distinct patterns of hybridization in the songbird forebrain. In addition, their sequences suggest relationships to known proteins, allowing some speculation about their potential roles in neural function. The characterization of each cDNA is presented as a separate chapter in this dissertation.

Chapter 2. Materials and Methods

DNA sequence analysis

Complete sequence of the HAT cDNA inserts was determined in both directions using Sequenase reagents and protocols (United States Biochemical) and synthetic primers prepared on a PCR-Mate (Applied Biosystems). The FASTA set of programs (Pearson and Lipman, 1988) was used to compare the nucleotide and deduced amino acid sequences to those in the current releases of the GenBank and Dayhoff databases.

PCR-based analysis of 5' HAT-3 sequences

Oligonucleotide primers were synthesized on a PCR-Mate (Applied Biosystems) with specificity to sequences either at the 5' end of the original HAT-3 cDNA insert or to sequences flanking the insertion site in the library cloning vector lambda gt10. Library DNA was subjected to successive rounds of amplification in a thermal cycler (American Scientific Plastics), and several DNA products were isolated by agarose gel electrophoresis. A second oligonucleotide specific for 5' sequences in the original HAT-3 cDNA was used as a primer for dideoxy-sequencing of one of these PCR products. The sequence thus generated included not only sequence which overlapped with the 5' end of the original HAT-3 cDNA, but also 195 bp of HAT-3 sequence not represented in the original clone.

Preparation of canary and zebra finch RNAs

Zebra finches were housed on 12 hour day/12 hour night fixed light cycle in the aviary at the University of Illinois. Nests were monitored closely, allowing young birds to be identified and hatch dates assigned to the nearest half day. Polyadenylated RNAs were prepared from individual zebra finch forebrains collected from birds of known age ranging from 1-47 days, using the FastTrack mRNA isolation kit (Invitrogen). RNA concentration was calculated from the OD_{260} measurements, assuming 1 OD_{260} unit = 40 ng/μ Samples were similarly prepared from tissues of canaries maintained on the natural photoperiod of New York state, harvested and frozen in October, 1990. These frozen tissus included lung, liver, heart, kidney, skeletal muscle, testes, and forebrain.

RNA Northern analysis of tissue-specific and developmental samples

RNA aliquots (above, 1 μg each) were electrophoresed on paraformaldehyde-agarose gels and transferred overnight with 10X SSC to Immobilon-N membranes (Millipore), following manufacturers suggestions for prewetting of membranes. RNAs were crosslinked to the membranes by UV irradiation, and preincubated with hybridization solution (263 mM NaHPO4, 10mM NaCl, 1mM EDTA, and 20 μg/ml polyA) in a roller oven for 0.5-2 hours at 65°C. HAT cDNA inserts were ^{32}P-labelled by the random priming method (Feinberg and Vogelstein, 1984), and probe was added to the hybridizations at 10^6 cpm/ml. Blots were hybridized overnight at

65° C, and washed repeatedly with 1X SSC, 1% SDS at 65° C before exposure to X-ray film (Kodak XAR).

In situ hybridization

Adult male canaries were sacrificed by decapitation. Whole brains were dissected and immediately frozen in OCT compound (Tissue-Tek), and 10μm sagittal sections were cut on a cryostat and mounted on organosilanated slides (Clayton and Alvarez-Buylla, 1989), fixed in PBS-buffered 4% paraformaldehyde (Clayton, et al., 1988), and stored at -70°C. Sections were hybridized to ^{35}S-labelled riboprobes using high stringency conditions (final wash: 11 mM sodium at 65°C) and exposed to X-ray film (Kodak XAR) (Clayton, et al., 1988). Some sections were dipped in Kodak NTB-2 emulsion, exposed, developed, and counterstained with cresyl violet following standard conditions (Rogers, 1979). Parallel hybridizations were performed with control probes for RNAs with defined distributions and with sense-strand controls for evaluation of non-specific background levels. Identification of song nuclei on X-ray films was confirmed by subsequent microscopic inspection of counterstained sections following emulsion autoradiography, with a canary stereotaxic atlas (Stokes, et al., 1974) as an anatomical reference.

Brains from zebra finches of known age were prepared as above and hybridized to ^{35}S-labelled riboprobes. Final wash conditions were 0.1X SSPE, 0.1X SDS.

Preparation of anti-peptide antibodies

Geneworks software (Intelligenetics) was used to identify regions within the predicted HAT proteins with high surface

probability, and peptides corresponding to these domains were synthesized by the Biotechnology Center at the University of Illinois. Each synthetic peptide was conjugated to the carrier molecule KLH (keyhole limpet hemocyanin) and purified by gel exclusion chromatography (Imject Activated Immunogen Conjugation Kit, Pierce). The Hybridoma Facility at the University of Illinois immunized mice and provided test sera. These sera were screened for specificity on dot blots representing increasing dilutions of immobilized target antigen. Antisera to HAT-14 peptides were screened against the specific peptide coupled to a carrier protein (BSA) which is distinct from the carrier (KLH) used in immunizations. HAT-3 sera were screened against a HAT-3 fusion protein generated in the pMAL bacterial expression system (New England Biolabs). The Hybridoma Lab at the University of Illinois produced polyclonal ascites fluid for all positively reactive animals. Crude ascites fluids were partially purified by precipitation with dextran sulfate and ammonium acetate (Harlow and Lane, 1988), dialyzed against PBS, aliquotted with 0.02% sodium azide, and stored at -20 C.

Preparation of protein extracts

A crude synaptosomal fractionation was performed on fresh tissue representing zebra finch forebrain (primarily telencephalon) and non-forebrain (cerebellum, optic tectum, brainstem, and spinal cord), and fresh or frozen tissue representing rat visual cortex or hippocampus as described (Oyler, et al., 1989). Briefly, tissues were homogenized in a Dounce homogenizer in ice-cold 0.32 M sucrose, 5 mM Tris, pH 7.5, 2.5 mM PMSF and centrifuged at 1000xg for 5

minutes at 4° C. The pellet was discarded, and the supernatant centrifuged at 12,500 X g for 15 minutes at 4°C. The supernatant (S2) was saved as the cytosolic fraction. The pellet (P2) was washed with homogenization buffer several times, pelleted again at 12,500 X g, and resuspended in homogenization buffer as the synaptosomal fraction. All protein samples were quantitated using the BioRad Protein Assay (Biorad), diluted to a convenient concentration, boiled in the presence of SDS sample buffer (Laemmli, 1970), and stored at -20° C.

Western blot analysis of protein

Proteins were separated by SDS-PAGE under standard Laemmli buffer conditions (Laemmli, 1970), and electrophoretically transferred to nitrocellulose (Hybond ECL, Amersham) for 1 hour at 100 V, in Towbin buffer with 20% methanol (Towbin, et al., 1979). Blots were then subjected to the following incubations: 5% nonfat dry milk (NFDM) in Tris-buffered saline with .1% Tween 20 (TBS-T) for 1 hour, TBS-T wash, primary antibody diluted in 1% NFDM in TBS-T for 1 hour, TBS-T wash, secondary antibody diluted in 1% NFDM in TBS-T for 1 hour, and a final wash in TBS-T. The blots were processed for chemiluminescence using ECL reagents (Amersham) and exposed to X-ray film.

Preabsorbed control antibodies were prepared in the following fashion. 1:1000 dilutions of antibodies in 5% NFDM TBS-T were incubated overnight at 4° C with either their specifically-recognized peptide or an unrelated peptide. These solutions were centrifuged at

1000 X g, and the supernatants used to probe identical blots of tissue extracts.

Perfusion fixation and PEG embedding of brain tissue for immunocytochemistry

Zebra finches are deeply anesthetized with Nembutol, then intracardially perfused with 10 ml PBS, followed by 20-40 ml of fixative (see below). Brains were post-fixed for 30 min.-2 hours, rinsed 3 times in PBS over 1 hour, and dehydrated in sequential ethanol baths of 50% (1 hour), 70% (1 hour), 95% (1 hour), and 100% (45 min.). Brains were then transferred to polyethylene glycol (PEG) baths maintained in a desiccator within a 46° C oven. Incubation was in successive baths of the indicated compositions: PEG 1000, ≥1 hour; PEG 1000/1540 (1:1 mixture), ≥1 hour; PEG1000/1540 (1:1 mixture), overnight; PEG 1540, 15 minutes. Tissue was then placed in an embedding mold (Peel-A-Way) with PEG 1540 and cooled for 2-5 minutes in a -20°C freezer. Solidified block were stored for days to weeks in sealed containers containing desiccant (Drierite) prior to sectioning on a rotary microtome. Ribbons of sections thus generated were stored for days to weeks in flat, shallow boxes, themselves stacked and sealed inside plastic bags containing desiccant and maintained in a cold room. Before staining, the sections were floated in PBS, mounted onto organosilanated slides, and dried briefly at room temperature (adapted from Clayton and Alvarez-Buylla, 1989).

Optimal fixation conditions varied with the antigen to be detected. HAT-14 immunoreactivity was most easily detected in brains fixed with 2% neutral-buffered paraformaldehyde

supplemented with .15% picric acid. HAT-3 immunoreactivity was best preserved by a solution of 3% neutral-buffered paraformaldehyde with .75M lysine and .1M sodium metaperiodate. Neither antigen was well-preserved in either 3% or 4% neutral-buffered paraformaldehyde alone.

Primary culture of zebra finch telencephalic tissue

Preparation of primary cultures from zebra finch telencephalon was based on the method of Baughman, et al., (Baughman, et al., 1991). Briefly, zebra finches from 1-15 days post hatch were injected with 50 microliters of a 10 mg/ml solution of ketamine. After a few minutes, the animals were decapitated, and their brains dissected into the following dissociation medium: minimal essential medium (MEM) with .35% glucose, 1mM kynurenic acid, 0.05mM APV (2-amino-phosphonovaleric acid), 0.5mM glutamine, and .026 M $NaHCO_3$, supplemented with 20 U/ml papain. The tissue was minced with fine scissors, then incubated on a rocker inside a 37 C oven for 30 minutes. The solution was triturated with a 5ml pipet, then subjected to 3 cycles of centrifugation (300 x g for 5 min.) followed by resuspension in the wash solution (prepared just as the dissociation solution, but ommitting the papain). After the final wash, the cells are resuspended in in growth medium (wash medium with 5% fetal bovine serum) and plated onto coverslips resting in the bottom of a multi-well dish.

Coverslips are prepared the night before by dipping in ethanol and flaming to ensure sterility, then incubating with 140 ul of a

solution of poly-D-lysine and laminin (.1mg/ml and .033mg/ml respectively).

Cultures were supplemented with fresh growth medium as needed every 3-5 days. Cultures to be stained were rinsed in PBS, then immersed in 50% methanol/50% acetone for 2 min. at room temperature. Coverslips were rinsed again in PBS, and kept immersed in PBS prior to immunostaining.

Immunocytochemistry

Mounted tissue sections were incubated with 0.2% Triton-X-100 in PBS for 5 minutes, then washed 4X in PBS over 20 minutes. Sections were blocked with normal goat serum for 30 minutes. The serum was gently blotted away, and a 1:100 dilution of polyclonal H3-1 or H14-c polyclonal ascites was applied for 2 hours. Sections were washed 5X over 25 minutes, then incubated with a 1:100 dilution of fluorescein-conjugated goat anti-mouse secondary antibody (Sigma). After 2 hours, the sections were again washed thoroughly in PBS, then coverslipped with aqueous mounting medium (Mowiol) and allowed to dry before visualization by fluorescence microscopy.

Primary cultures were stained with HAT-3 reactive ascites as detailed above, except that permeabilization of the tissue by Triton was unnecessary. Incubation times were likewise shortened (30 min. - 1 hour) for the primary and secondary antibodies. Non-immunize ascites were applied at similar dilutions in place of the primary antibody as a control for non-specific staining. After the

final wash, coverslips were mounted in aqueous medium (Mowiol) and allowed to dry.

Chapter 3. HAT-2 encodes a novel signal transduction protein and is differentially regulated within the song control circuit.

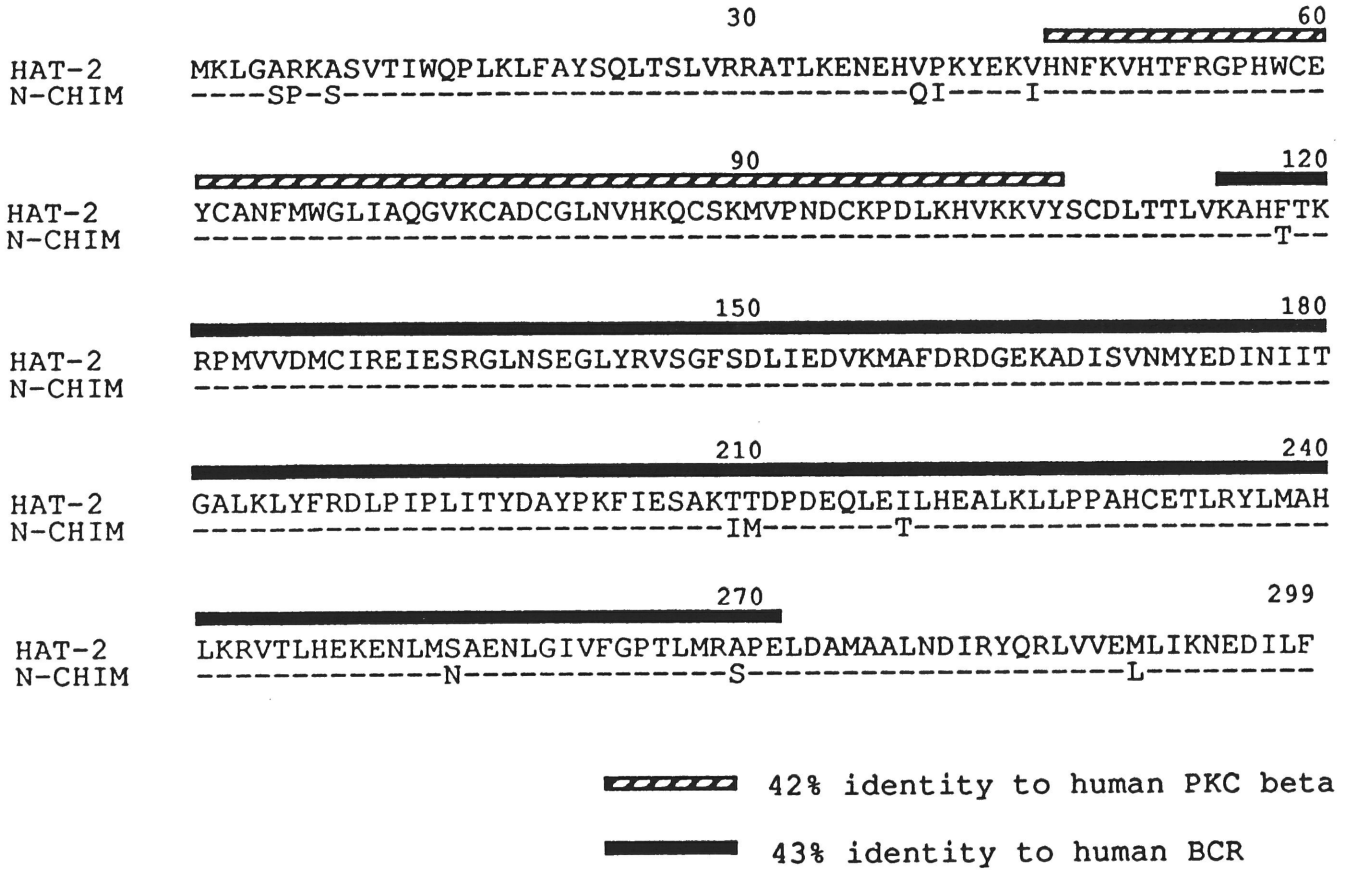
HAT-2 encodes a protein with homology to protein kinase C and the product of the *bcr* oncogene

The nucleic acid sequence of HAT-2 is presented in Fig. 2, along with the predicted protein sequence of 299 amino acids. This protein sequence was compared to sequences in the Dayhoff and Genbank protein databases using the FASTA algorithm (Pearson and Lipman, 1988) and two distinct regions of similarity to other molecules were identified (Fig. 3). Amino acids 46-106 share ~40% identity with the domain mediating diacylglycerol regulation in many protein kinase C isotypes (Ono, et al., 1989). The cysteine consensus $C\text{-}X_2\text{-}C\text{-}X_{13}\text{-}C\text{-}X_2\text{-}C\text{-}X_7\text{-}C\text{-}X_7\text{-}C$, thought to generate a zinc-finger capable of phorbol ester binding in PKase C, is perfectly conserved in the HAT-2 sequence. Residues 115-270 in the HAT-2 protein share 43% identity with the product of the human breakpoint cluster region gene, *bcr* (Hariharan and Adams, 1987), (Heisterkamp, et al., 1985), (Lifshitz, et al., 1988), (Fig. 3).

FIGURE 2. Nucleotide sequence and predicted amino acid product of HAT-2 cDNA. HAT-2 represents 1695 bp of a ~2200 bp transcript in canary brain. The predicted amino acid sequence (one letter code) is identical in length (299 amino acids) to the product of human n-chimaerin cDNA, although the possible existience of additional protein-coding sequence in the missing 5' portion of the HAT-2 RNA cannot be rigorously excluded without a full-length cDNA. Conserved cysteines within the zinc finger domain believed to mediate phorbol ester binding (see text) are highlighted (#).

```
                                                  M  K  L  G  A  R  K  A        8
        CCGGCAGCGCGATCTGCTGCTCGCAGCCTTGGGCATGAAACTGGGCGCTCGCAAGGCG          58

  9  S  V  T  I  W  Q  P  L  K  L  F  A  Y  S  Q  L  T  S  L  V               28
 59  TCTGTGACAATCTGGCAACCTCTTAAACTCTTTGCTTATTCGCAGTTGACGTCGCTCGTC            118

 29  R  R  A  T  L  K  E  N  E  H  V  P  K  Y  E  K  V  H  N  F               48
119  CGAAGAGCAACTCTGAAGGAAAATGAACACGTTCCAAAATACGAGAAGGTTCATAATTTC            178
                                        #           #
 49  K  V  H  T  F  R  G  P  H  W  C  E  Y  C  A  N  F  M  W  G               68
179  AAGGTGCACACGTTCAGGGGCCCGCACTGGTGTGAATACTGTGCCAACTTCATGTGGGGC            238
                          #           #                       #
 69  L  I  A  Q  G  V  K  C  A  D  C  G  L  N  V  H  K  Q  C  S               88
239  CTCATTGCTCAGGGAGTAAAATGTGCAGATTGTGGTTTAAATGTGCACAAGCAATGTTCC            298
                       #
 89  K  M  V  P  N  D  C  K  P  D  L  K  H  V  K  K  V  Y  S  C              108
299  AAGATGGTCCCAAATGACTGCAAGCCAGACCTGAAGCATGTCAAAAAAGTGTACAGCTGT            358

109  D  L  T  T  L  V  K  A  H  F  T  K  R  P  M  V  V  D  M  C              128
359  GACCTTACCACGCTAGTAAAAGCACACTTCACCAAGAGACCAATGGTGGTAGATATGTGC            418

129  I  R  E  I  E  S  R  G  L  N  S  E  G  L  Y  R  V  S  G  F              148
419  ATTAGGGAAATTGAATCTAGAGGTCTTAATTCTGAAGGACTGTACCGAGTCTCAGGATTT            478

149  S  D  L  I  E  D  V  K  M  A  F  D  R  D  G  E  K  A  D  I              168
479  AGTGATCTTATTGAAGATGTCAAAATGGCGTTTGACAGAGATGGGGAAAAAGCTGATATT            538

169  S  V  N  M  Y  E  D  I  N  I  I  T  G  A  L  K  L  Y  F  R              188
539  TCTGTGAATATGTATGAAGATATCAATATTATCACTGGTGCACTTAAACTGTACTTCAGG            598

189  D  L  P  I  P  L  I  T  Y  D  A  Y  P  K  F  I  E  S  A  K              208
599  GATTTGCCAATTCCACTCATCACATATGATGCCTACCCAAAGTTCATAGAGTCTGCAAAA            658

209  T  T  D  P  D  E  Q  L  E  I  L  H  E  A  L  K  L  L  P  P              228
659  ACCACGGATCCGGATGAACAGCTGGAAATTCTCCACGAGGCGCTGAAGCTGCTGCCGCCT            718

229  A  H  C  E  T  L  R  Y  L  M  A  H  L  K  R  V  T  L  H  E              248
719  GCACACTGTGAAACTTTACGGTATCTCATGGCACATCTGAAGAGAGTAACACTCCATGAA            778

249  K  E  N  L  M  S  A  E  N  L  G  I  V  F  G  P  T  L  M  R              268
779  AAGGAAAACCTGATGAGTGCAGAAAACCTGGGCATAGTTTTTGGACCAACTCTTATGAGA            838

269  A  P  E  L  D  A  M  A  A  L  N  D  I  R  Y  Q  R  L  V  V              288
839  GCGCCGGAGCTGGACGCGATGGCTGCGCTGAACGACATCCGCTATCAGAGACTTGTGGTG            898

289  E  M  L  I  K  N  E  D  I  L  F
899  GAGATGCTTATAAAAAATGAAGACATTTTATTTTGAATATTTTGGGGGTTTTGTAATAAA            958
959  AAAGGAAGCAACAGTGTTCTATGGATGAAGGAATATTTTAAAGTAATTTAATGGCTCTTG           1018
1019 TCACTGAATTCCATATTTGCTAGAGCTTTCGATGTATTCAGGATACAAATGAAGGAACTC           1078
1079 TTTGTCGTTTCTGTAGTGCCATTCAGCTGATGTTGAAAAAGGTTAACACATACTTTCCAG           1138
1139 TACTAGTAATCCTGGGTGTTTATCATGTTGAATTAAAAAACAAAATGCCTAAAGCTATTG           1198
1199 CATGATTTGCTCCCTGTTCTCCCTGTTCTGGTGAGACTCATTCCATGAAGAAAACAACTG           1258
1259 AACTGGTGCAGCTATCTTTGTTCTGGATAGTTTGTGATTGTAATTCAGCATGTTTCTCCG           1318
1319 TGTAAACCTGTTGTGAATTTGCTTTTTTGTTCTATGTAATTGGTTTCTAATACTACAAAG           1378
1379 AAGGCAGACTTCTGTGAAATGGAGCCTCTGGCCATTTATTGACATTTCATATTCTTCTCT           1438
1439 TCCACTTCTGGGGCTGATTTATTCTTGGGTTTCTTTCCATTTTTTCATCATATAGTAATT           1498
1499 TGTTTTTAACAGAAACAAAATTGTACTTTAAATGTTACTTTAAGTAATTCTCCATGATGT           1558
1559 TTATGGTGGTTGCAGTGAAATCTGCAATACTGAACAGTGTTCCTTTATTATTATTGCTAT           1618
1619 TTCAATTGTAATTTTGTATTTTTATCTGGCATGCATATATTAATTTATTAAATTTTGCTT           1678
1679 TTAGAACTCTAAAAAAA
```

FIGURE 3. Alignment of canary HAT-2 protein and human n-chimaerin, with domains of similarity to PKaseC and BCR. Human and canary sequences are aligned, demonstrating 96% amino acid identity overall. Dashes (-) in the n-chimaerin sequence indicate identical residues; substitutions are noted. An N-terminal domain with 42% identity to the C1 regulatory region of PKaseC is indicated with a cross-hatched bar above. A domain with 43% identity to the C-terminus of BCR, the product of the breakpoint cluster region gene, is indicated with a solid bar above.


30
60
HAT-2 MKLGARKASVTIWQPLKLFAYSQLTSLVRRATLKENEHVPKYEKVHNFKVHTFRGPHWCE
N-CHIM ----SP-S----------QI----I----------
90
120
HAT-2 YCANFMWGLIAQGVKCADCGLNVHKQCSKMVPNDCKPDLKHVKKVYSCDLTTLVKAHFTK
N-CHIM ----------T--
150
180
HAT-2 RPMVVDMCIREIESRGLNSEGLYRVSGFSDLIEDVKMAFDRDGEKADISVNMYEDINIIT
N-CHIM ----------
210
240
HAT-2 GALKLYFRDLPIPLITYDAYPKFIESAKTTDPDEQLEILHEALKLLPPAHCETLRYLMAH
N-CHIM ----------IM-------T----------
270
299
HAT-2 LKRVTLHEKENLMSAENLGIVFGPTLMRAPELDAMAALNDIRYQRLVVEMLIKNEDILF
N-CHIM ----------N----------S----------L---------
42% identity to human PKC beta
43% identity to human BCR

High conservation between the canary HAT-2 protein and a human sequence, n-chimaerin

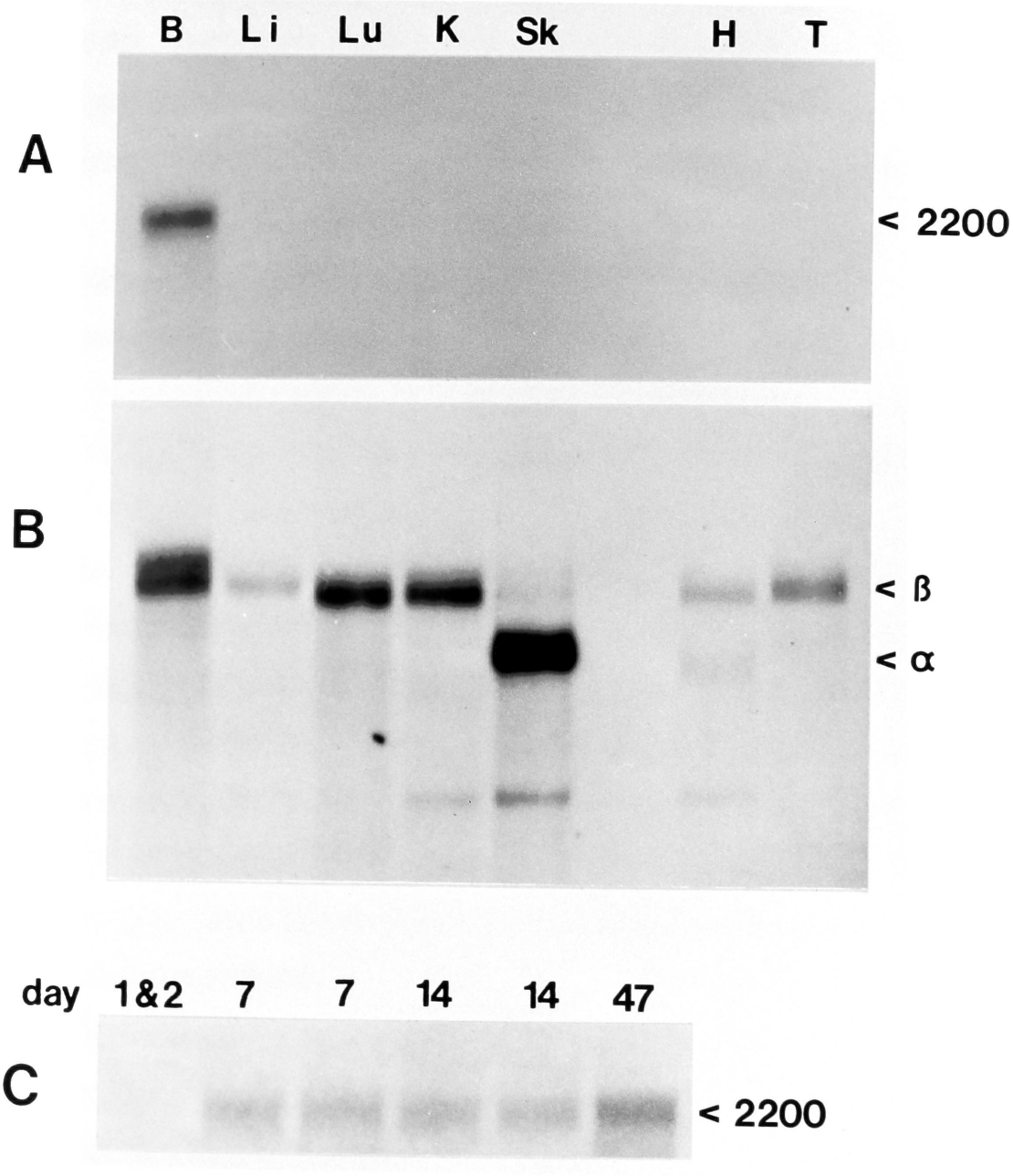
A recently described human sequence is likewise related to both the PKaseC zinc finger and BCR (Hall, et al., 1990a). Comparison of HAT-2 with this human sequence indicates that the two proteins are 96% identical (Fig. 3). Most of the 13 substitutions in the two amino acid sequences are conservative; 7 occur in the regions flanking the PKaseC- and BCR-like domains, and the remainder are within the BCR-like domain. The PKaseC-like domains in the two sequences are 100% identical. Conservation also extends into the 3' untranslated regions of the two RNAs, which are of similar size (HAT-2, 762 nucleotides; n-chimaerin, 729 nucleotides) and ~65% identical in sequence (the two coding regions share 87% nucleotide identity).

HAT-2 mRNA is brain-specific and developmentally regulated in zebra finches

The HAT-2 cDNA was used to probe Northern blots of poly(A)$^+$ RNA representing a variety of canary tissues including forebrain, liver, lung, kidney, skeletal muscle, heart, and testes (Fig. 4, Panel A). The HAT-2 transcript was detected only in brain. When the same blots were reprobed for actin (Panel B), the ubiquitous β-actin transcript was detected in all tissues, and additional actin isoforms were detected in the muscle tissue samples. It is concluded therefore that HAT-2 mRNA is specific to brain tissue.

FIGURE 4. Northern analysis of HAT-2 mRNA in canary tissues and developing zebra finch forebrain. **Panel A:** ^{32}P-labelled cDNA probe from the HAT-2 clone hybridizes to a dominant band of ~2200 bp only in zebra finch brain. Tissues of RNA origin (l. to r.) include forebrain, liver, lung, kidney, skeletal muscle, (empty lane), heart, and testes. **Panel B:** The same blot reprobed with ^{32}P-labelled cDNA probe from chicken βactin clone. Positions of α and βisoforms are indicated.

Panel C: Developmental profile of RNA expression. Each lane represents poly(A)+ RNA isolated from the forebrain of a single different zebra finch of the indicated age, except for the 1-2 day sample, in which a 1-day and a 2-day sample were pooled.


B
Li
Lu
K
Sk
H
T
A
< 2200
B
< ß
< α
day
1&2
7
7
14
14
47
C
< 2200

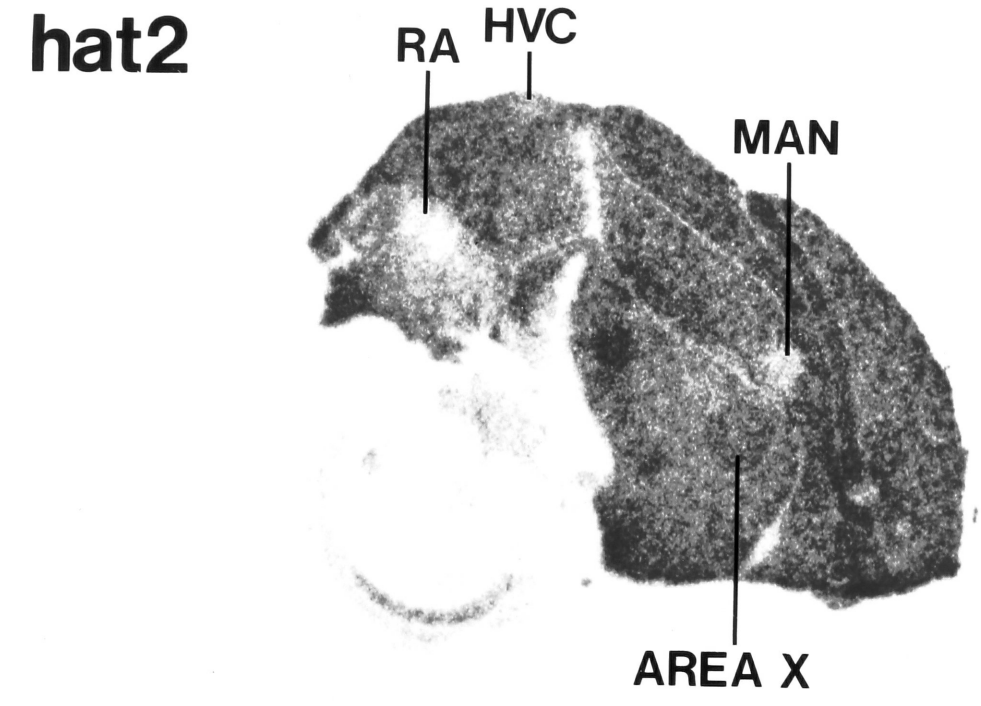
Northern analysis was similarly applied to determine the developmental onset of HAT-2 mRNA expression in young zebra finches. Poly $(A)^+$ RNA was isolated from the forebrains of zebra finches of known age, blotted, and probed with the HAT-2 cDNA (Fig. 4, Panel B). Very little HAT-2 RNA is detected at 1-2 days, but by 7 days expression has nearly reached the level observed in adult birds. Apparently, levels of HAT-2 mRNA rise sometime in the first week post-hatch, a period otherwise characterized by high levels of synaptogenesis (Herrmann and Bischof, 1986) and a near quadrupling of brain mass (J. George, unpublished observation).

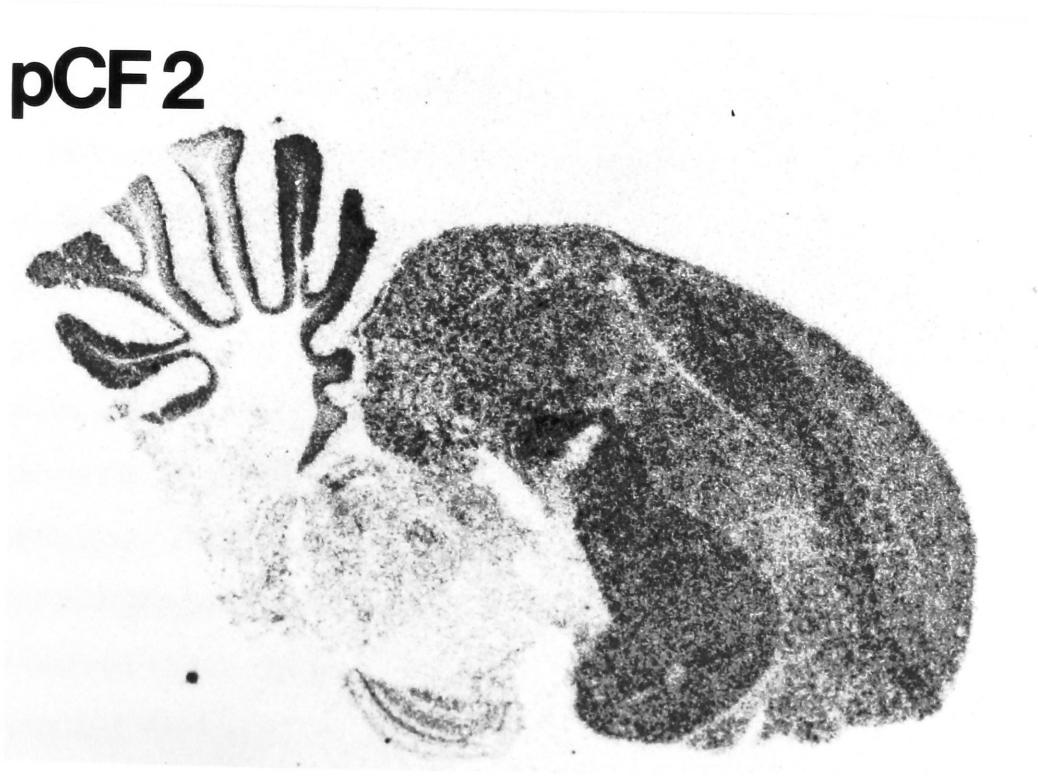
HAT-2 mRNA is differentially expressed in the song circuit

The HAT-2 RNA was localized in the forebrain using in situ hybridization. (Fig. 5). Brain sections from male canaries in Spring (when androgen levels and song production are high, but song learning has ceased (Nottebohm, 1987a)) were hybridized with ^{35}S-labelled riboprobes, washed at high stringency, and exposed to X-ray film. The resulting hybridization signal is high throughout most of the forebrain, much lower in the midbrain and tectum, and virtually absent in the cerebellum.

Within the forebrain, HAT-2 RNA is notably lower in fiber tracts and in song control nuclei HVC, RA and lateral MAN, but more abundant in a fourth song nucleus, Area X, compared to the regions in which these nuclei sit. Control RNAs do not distinguish the song nuclei from surrounding brain regions (Fig. 5, lower panel). Thus variations in HAT-2 within the song

FIGURE 5. Distribution of HAT-2 mRNA in sagittal sections of adult male canary brain. **A:** in situ hybridization pattern for HAT-2 probe. Song control nuclei HVC, RA, lateral MAN, and Area X are indicated with line segments (refer to Fig. 1). Presence of song control nuclei was verified by light microscopic examination of cresyl violet counterstained tissue. Cerebellum and brainstem are present but only barely visible due to the low hybridization signal in these regions (see B). **B:** hybridization pattern for control probe pCF2 (Clayton et al., 1988). This probe does not distinguish song nuclei from surrounding tissue, although their presence in this section was confirmed by examination of counterstained tissue.


hat2
RA
HVC
MAN
AREA X


pCF 2

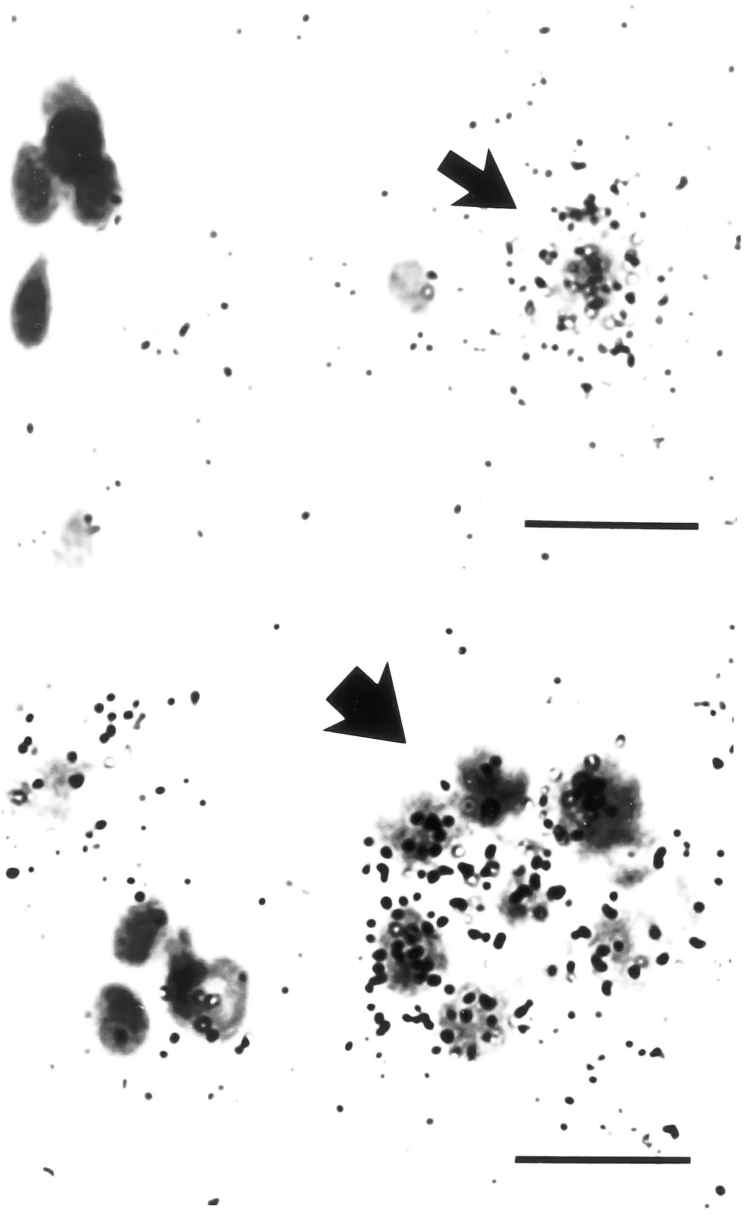
circuit presumably reflect specific gene regulation, as opposed to non-specific factors such as cell density or metabolic activity. By densitometric analysis of X-ray film images from birds taken throughout the Spring, these song circuit variations fall within a 2-fold range above or below surrounding brain regions.

Patterns of HAT-2 RNA distribution were also compared in male canaries taken in the Fall (October) when singing behavior is more plastic. No obvious differences were observed in the song nuclei of these birds as compared to Spring birds (data not shown).

HAT-2 mRNA is expressed in neurons in the song control circuit

To determine the cell types that express *hat-2* in parts of the song circuit where regulation may be occurring, hybridized sections were exposed to autoradiographic emulsion and counterstained with cresyl violet. Song nucleus RA contains large distinctive neurons, and these are preferentially labelled by the *hat-2* probe (Fig. 6). In song nucleus HVC, cells are typically organized into clusters containing a large central Area X-projecting neuron surrounded by a number of smaller cells, some or all of which are RA-projecting neurons (Burd and Nottebohm, 1985), (Kirn and Nottebohm, 1990). Autoradiographic silver grains are present over most or all of the cells in these clusters (lower panel, arrow). It is therefore concluded that *hat-2* is expressed in neurons, and probably in a variety of functional subclasses. Similar conclusions were

FIGURE 6. Cellular expression of HAT-2 mRNA in song nuclei RA and HVC (bright-field emulsion autoradiographs). **Upper panel:** in RA, grains are concentrated primarily over large isolated cell bodies with neuronal morphology. **Lower panel:** in HVC, concentrations of autoradiographic grains are seen across a cell cluster typical of this nucleus (Kirn and Nottebohm, 1990) (arrowhead), including the large central neuron. By comparison, other cDNA probes (Clayton et al., 1988) label only the surrounding cells and not the central neuron (not shown). Negligible silver grains were generated in control hybridizations using sense-strand probes (not shown). Bar size = 12 μm.



reached regarding the distribution of n-chimaerin RNA in the mammalian nervous system (Hall, et al., 1990). The present data do not address whether *hat-2* may also be expressed in glia.

Discussion

The canary HAT-2 cDNA predicts an unusual protein which, because of its conserved structure and regulation, is likely of fundamental importance to the vertebrate forebrain. A cDNA with very similar sequence has also been isolated from human brain, and the predicted protein was named n-chimaerin in reference to the presence of two discrete domains of similarity to other proteins, protein kinase C and the *bcr* oncogene (Ahmed, et al., 1990). The PKaseC-like domain includes precisely one of the cysteine rich 'zinc finger' elements found in the C1 region of PKaseC (Nishizuka, 1988), and known to confer diacylglycerol and phorbol ester binding (Ono, et al., 1989). This domain of n-chimaerin has been demonstrated to bind phorbol ester (Ahmed, et al., 1990) in a zinc-dependent fashion (Ahmed, et al., 1991). This raises the interesting possibility of an independent diacylglycerol signal transduction pathway through HAT-2/n-chimaerin that parallels or complements the PKaseC pathway. A variety of experiments have implicated PKaseC in long-term memory formation, using assays such as phorbol ester binding (Olds, et al., 1989), or inhibitors of kinase activation (Malenka, et al., 1989). Many of these approaches may not necessarily distinguish between PKaseC and other peptides such as HAT-2 which share potential diacylglycerol-binding regulatory domains. Highly specific

pharmacological agents, e.g. peptide pseudosubstrate antagonists (House and Kemp, 1987)(Malinow, et al., 1989), will therefore be required to discriminate between effects involving these related molecules.

In protein kinase C, the region adjacent to the diacylglycerol binding regulatory domain comprises the catalytic kinase domain. In HAT-2/n-chimaerin, this adjacent region is related instead to BCR, a protein whose function is yet unknown. BCR has been reported to have kinase activity (Timmons and Witte, 1989), although it bears none of the structural features considered diagnostic of the protein kinase family (Hanks, et al., 1988). The *bcr* gene was identified through its participation in pathological gene fusion events associated with human chronic myelogenous leukemia (CML) (Shtivelman, et al., 1985). The molecular defect seems to be activation of the oncogene abl upon upstream insertion of a 5' fragment of *bcr* (Konopka, et al., 1984). However, HAT-2 shares homology with a 3' fragment of *bcr* which is completely distinct from the fragment that fuses with *abl* (Fig. 3).

Recently, C-terminal portions of the BCR and n-chimaerin proteins were shown *in vitro* to regulate the GTPase activity of $p21^{rac}$, a guanine nucleotide binding protein related to the oncoprotein RAS (Diekmann, et al., 1991). This C-terminal domain was also shown to have structural similarity to other GTPase-activating proteins (GAPs) in the $p21^{rho}$ family (Diekmann, et al., 1991). Guanine nucleotide binding proteins and their regulators appear to be involved in a wide variety of neural processes, possibly including long-term potentiation (Simon, et al., 1991).

Although the relationship of HAT-2/n-chimaerin with PKaseC and BCR is compelling, the level of conservation between canary HAT-2 and human n-chimaerin (96% amino acid identity) is much higher than the conservation between either of these sequences and PKaseC or BCR (40% identity within the respective domains). In fact, HAT-2/n-chimaerin is among the more highly conserved of those proteins for which avian and human sequences have been compared (Nei, 1987). These observations suggest that the protein has evolved to serve a discrete and stable role in the vertebrate nervous system, related to but clearly distinct from the function of its protein kinase C and BCR relatives.

Not only has molecular sequence been conserved between HAT-2 and n-chimaerin, but the basic pattern of expression in the brain is also similar in birds and mammals. The HAT-2 RNA is highly enriched in 'striatal' regions of the canary forebrain, and n-chimaerin RNA is enriched in cortical regions of the mammalian forebrain (Ahmed, et al., 1990). These parts of the avian and mammalian brain are considered to be functionally and evolutionarily homologous, though very different in organization, and are involved in the control of complex learned behaviors and higher perceptual processes (Benowitz, 1980)(Northcutt, 1981)(Reiner, 1986)(Saini and Leppelsack, 1981). In both birds and mammals, the RNA is much less abundant in other parts of the brain. These observations suggest that HAT-2 contributes to special properties of the forebrain that are shared widely among vertebrates despite differences in forebrain organization.

hat-2 gene expression is relatively homogeneous throughout most of the canary forebrain, but varies substantially within the song control circuit of the Spring male canaries examined here. The RNA is less abundant in HVC, RA, and lateral MAN, all of which are known to bind androgen hormones (Arnold, 1985a). The *hat-2* RNA is relatively more abundant in Area X, which does not display detectable androgen binding activity (Arnold, 1980a). Androgen levels are high in Spring and are believed to influence cellular properties in the song nuclei associated with neuronal growth and song stabilization (Marler, et al., 1988). This suggested the hypothesis that the *hat-2* gene is subject to repression by androgens, with variations in its expression contributing to the seasonal changes in song learning and behavior and song circuit anatomy. However, no obvious differences were observed between HAT-2 hybridization patterns among Spring (March-May) and Fall (October) canaries. Transient changes in gene expression might have been missed, however, in this small sample (7 Spring and 4 Fall birds), and sampling at other times during the year will be necessary before concluding that such a change in *hat-2* expression does not occur.

Chapter 4. HAT-5 encodes the homolog of MAP kinase-kinase, a protein implicated in growth factor signal transduction

HAT-5 predicts a protein kinase

Another HAT-enriched mRNA identified by the differential cDNA cloning techniques described in Chapter 1 is HAT-5. The 1977 bp cDNA insert hybridizes to an mRNA species of approximately 2700 bp on Northern blots of poly(A)$^+$ RNA from canary brain (Table 1). This RNA is enriched $\geq$3-fold in the HAT relative to non-forebrain, and is present in the HAT at 10^{-5}-10^{-4} as a fraction of total polyadenylated RNA mass (Table 1).

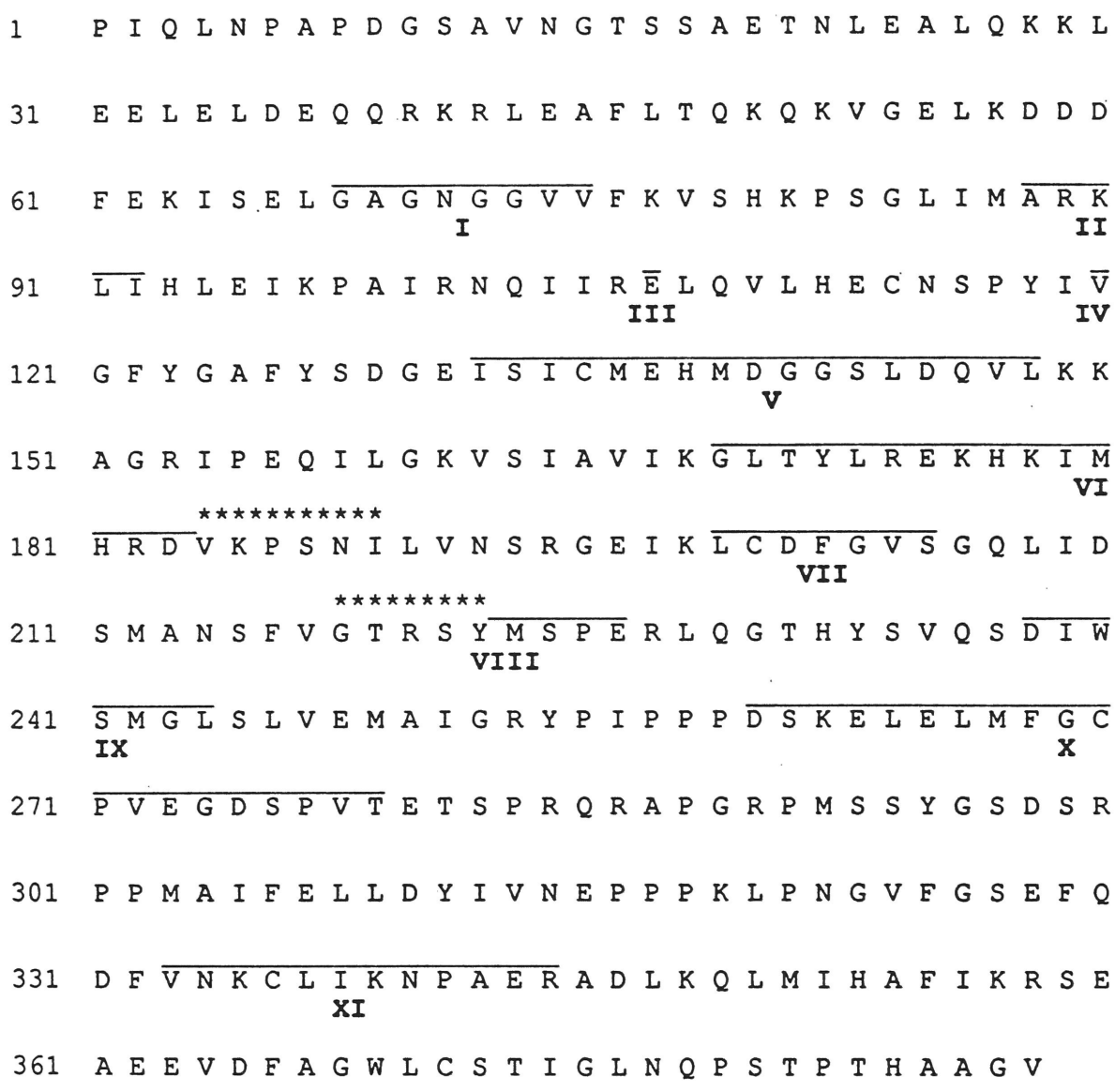
The nucleic acid sequence of canary HAT-5 is shown in Figure 7, along with the peptide predicted by the longest open reading frame, extending from the 5' end of the cDNA to a stop codon at bp 1167. When the 388 residue peptide was compared to those present in the GenBank and Dayhoff protein databases, similarity was found to a number of protein kinases. Further analysis of the HAT-5 sequence revealed the presence of all of the 11 domains reported to be diagnostic for members of the protein kinase family (Hanks, et al., 1988), (Fig. 8).

In initial comparisons, HAT-5 was found to be most related to a group of yeast kinases, including BYR1, STE7, and PBS2. BYR1 and STE7 are homologs in the yeast species *Saccharomyces pombe* and *Saccharomyces cerevisiae*, respectively, and are thought to function downstream of *ras*

FIGURE 7. Nucleotide sequence and predicted amino acid product of HAT-5 cDNA. HAT-5 represents 1977 bp of a 2700 bp transcript in brain. The longest open reading frame includes 388 amino acids (indicated by one-letter code), although additional N-terminal residues including a starting methionine are probably represented in 5' sequence which is missing from this clone.

```
GGCCGATCCAGCTGAACCCCGCTCCGGATGGATCCGCCGTGAACGGGACCAGCTCTGCGGAGACAAATCTGGAGG   75
  P  I  Q  L  N  P  A  P  D  G  S  A  V  N  G  T  S  S  A  E  T  N  L  E  A

CCCTTCAGAAGAAGCTGGAGGAGCTGGAGCTGGATGAGCAGCAGCGGAAGCGCCTGGAGGCGTTCCTCACCCAGA  150
  L  Q  K  K  L  E  E  L  E  L  D  E  Q  Q  R  K  R  L  E  A  F  L  T  Q  K

AACAGAAGGTTGGGGAGCTGAAGGATGATGACTTTGAGAAGATCAGTGAGCTGGGAGCAGGCAATGGTGGTGTGG  225
  Q  K  V  G  E  L  K  D  D  D  F  E  K  I  S  E  L  G  A  G  N  G  G  V  V

TCTTCAAAGTGTCTCACAAACCCTCTGGCCTCATCATGGCAAGAAAGTTAATCCACCTGGAGATCAAGCCAGCCA  300
  F  K  V  S  H  K  P  S  G  L  I  M  A  R  K  L  I  H  L  E  I  K  P  A  I

TCCGCAACCAGATCATCCGGGAGCTGCAGGTGCTGCACGAGTGCAACTCCCCCTACATCGTGGGCTTCTATGGAG  375
  R  N  Q  I  I  R  E  L  Q  V  L  H  E  C  N  S  P  Y  I  V  G  F  Y  G  A

CCTTCTACAGTGATGGGGAGATCTCCATCTGCATGGAGCACATGGATGGTGGCTCCTTGGATCAAGTGCTGAAAA  450
  F  Y  S  D  G  E  I  S  I  C  M  E  H  M  D  G  G  S  L  D  Q  V  L  K  K

AGGCTGGGAGGATTCCAGAGCAGATCCTGGGCAAAGTTAGCATTGCGGTAATAAAAGGACTCACATATCTGAGAG  525
  A  G  R  I  P  E  Q  I  L  G  K  V  S  I  A  V  I  K  G  L  T  Y  L  R  E

AAAAACATAAAATAATGCACAGAGATGTAAAACCATCCAACATTTTGGTAAACTCTAGAGGTGAAATCAAGCTTT  600
  K  H  K  I  M  H  R  D  V  K  P  S  N  I  L  V  N  S  R  G  E  I  K  L  C

GTGACTTTGGTGTCAGTGGGCAGCTGATAGATTCCATGGCAAACTCCTTTGTTGGCACACGCTCCTACATGTCTC  675
  D  F  G  V  S  G  Q  L  I  D  S  M  A  N  S  F  V  G  T  R  S  Y  M  S  P

CCGAGAGGCTGCAGGGCACTCACTACTCAGTGCAGTCAGACATCTGGAGCATGGGGCTGTCCCTGGTGGAGATGG  750
  E  R  L  Q  G  T  H  Y  S  V  Q  S  D  I  W  S  M  G  L  S  L  V  E  M  A

CCATTGGCAGGTACCCCATCCCCCCTCCTGACTCCAAGGAGCTGGAGCTGATGTTTGGCTGCCCCGTGGAGGGGG  825
  I  G  R  Y  P  I  P  P  P  D  S  K  E  L  E  L  M  F  G  C  P  V  E  G  D

ATTCTCCAGTCACAGAGACCTCGCCCAGGCAAAGAGCGCCCGGCCGGCCCATGAGCTCCTATGGATCAGACAGCA  900
  S  P  V  T  E  T  S  P  R  Q  R  A  P  G  R  P  M  S  S  Y  G  S  D  S  R

GACCCCCAATGGCAATCTTTGAACTTCTGGATTACATCGTCAATGAGCCACCTCCAAAACTGCCCAATGGTGTTT  975
  P  P  M  A  I  F  E  L  L  D  Y  I  V  N  E  P  P  P  K  L  P  N  G  V  F

TTGGTTCTGAATTTCAAGATTTTGTTAACAAATGTTTAATTAAAAATCCTGCTGAGAGAGCCGACTTGAAGCAGC 1050
  G  S  E  F  Q  D  F  V  N  K  C  L  I  K  N  P  A  E  R  A  D  L  K  Q  L

TGATGATCCATGCTTTCATCAAGAGATCCGAGGCGGAGGAGGTGGATTTTGCGGGGGTGGCTCTGCTCCACCATCG 1125
  M  I  H  A  F  I  K  R  S  E  A  E  E  V  D  F  A  G  W  L  C  S  T  I  G

GCCTTAACCAGCCCAGCACCCCCACGCACGCCGCCGGCGTCTGAGCCCGGGGGCCAGCTCCTGGACTGTACCTCTG 1200
  L  N  Q  P  S  T  P  T  H  A  A  G  V

GTGACAACCACGCTCTCCTGGTCCTCTCTCCTCAGCTTGTACCTGTTCCAACATGTATTTCACCTCTTGAGGAAG 1275
AATGTCTTGATAGCATGTGCCAAAACGGTTTTAAATCTCGTCCTAAACTAATTGGTATCGTTCCGGGTTCCGTCG 1350
TTCACTGACCAAATGTAAGCTGTGTAAGTTCCAGTGCTTGCTGATTTTAGGTGATTATGGATATTCTTTCTTAAT 1425
GAAAATATCACTGGGGCAGGGGGGGGTTGGCCCCTGGCTTGTTTGAACTTTATCAAGACTCTTTGTAAATCGTTG 1500
GTACTTCAATCATGCTTTCCCTGAGCTCCCCGGGCTTGGGATCGGGATGCTCCGAGCCTGTCTGTGAGCATGCTT 1575
TGCTGCTGCCCAGCTGTTCCCAAAAGTTAGGCTCCAATTTTGCTGTTGTCTAGAGATCTCTCTCTTTGCAGGTGA 1650
AGAAGGCAAGAGCTCTGCATTTCTCAGAATGCACAGGGCAATATTCTAATTGTAGACTTGTTCATATTTCTATAT 1725
TTATTTTTCGAACATATCATCATCCTTGGATTTAGTGATGTATGTCTTTCTAATTGATTTTTAAAAGTTAGTTCT 1800
TGGAAGTGTCCTATGGAATCAAGACAATGATCCAAGCACTTTGCTTTCTTTTAACCTAAAGAGTCATGACAACTG 1875
TGTTTAGTGTCTAAAAGTGTATGAAAATCCTTTATTGTTTTATTCTCTCAGATGTTTAGCAATGGGTTCTCTTAA 1950
TAAATATATTATCAAGTAAAAAAAAAA                                                   1977
```

FIGURE 8. The predicted HAT-5 peptide with 11 domains diagnostic for protein kinases. The 388 residue HAT-5 peptide is indicated with the one-letter code. Domains number I-XI are indicated by underlining. This are regions are well-conserved among protein kinases. (******) indicate additional sequences characteristic of serine/threonine specific protein kinases (Hanks et al., 1988).


1 P I Q L N P A P D G S A V N G T S S A E T N L E A L Q K K L
31 E E L E L D E Q Q R K R L E A F L T Q K Q K V G E L K D D D
61 F E K I S E L G A G N G G V V F K V S H K P S G L I M A R K
I
II
91 L I H L E I K P A I R N Q I I R E L Q V L H E C N S P Y I V
III
IV
121 G F Y G A F Y S D G E I S I C M E H M D G G S L D Q V L K K
V
151 A G R I P E Q I L G K V S I A V I K G L T Y L R E K H K I M
VI
****
181 H R D V K P S N I L V N S R G E I K L C D F G V S G Q L I D
VII
****
211 S M A N S F V G T R S Y M S P E R L Q G T H Y S V Q S D I W
VIII
241 S M G L S L V E M A I G R Y P I P P P D S K E L E L M F G C
IX
X
271 P V E G D S P V T E T S P R Q R A P G R P M S S Y G S D S R
301 P P M A I F E L L D Y I V N E P P P K L P N G V F G S E F Q
331 D F V N K C L I K N P A E R A D L K Q L M I H A F I K R S E
XI
361 A E E V D F A G W L C S T I G L N Q P S T P T H A A G V

in signal transduction cascades influencing cell-type determination (Nadin-Davis and Nasim, 1988), (Teague, et al., 1986). PBS2 confers polymyxin B resistance to *Saccharomyces cerevisiae* by an unknown mechanism (Boguslawski and Polazzi, 1987). Vertebrate kinases such as cAMP dependent kinases, protein kinase C, Ca^{++}/calmodulin dependent kinases, and various receptor kinases were also present in the databases, but displayed significantly less relationship to HAT-5.

HAT-5 encodes the canary homolog of MAP kinase-kinase

Figure 9 displays the alignment of HAT-5 with the recently reported murine sequence denoted MEK1, for MAP kinase or ERK kinase (Crews, et al., 1992). The two predicted peptides share 95% sequence identity within the overlapping regions. While the HAT-5 cDNA does not extend 5' to the presumed initiating methionine, its predicted peptide includes all but the 7 amino terminal residues encoded by the murine clone.

HAT-5 RNA is enriched in songbird brain

The HAT-5 cDNA was used to probe Northern blots of poly(A)$^+$ RNA representing a variety of canary tissues (Fig. 10, upper panel). Identical blots were probed with β-actin as a control for differential RNA degradation (see Ch. 3). The HAT-5 transcript was found to be enriched in brain, but expressed in lung and kidney at relatively high levels as well.

FIGURE 9. Alignment of canary HAT-5 protein and murine MEK1. Identical residues are boxed; non-identical residues generally represent conservative substitutions. MEK1 includes 7 N-terminal residues not encoded by the HAT-5 cDNA. The two sequences are 95% identical in the region of overlap.

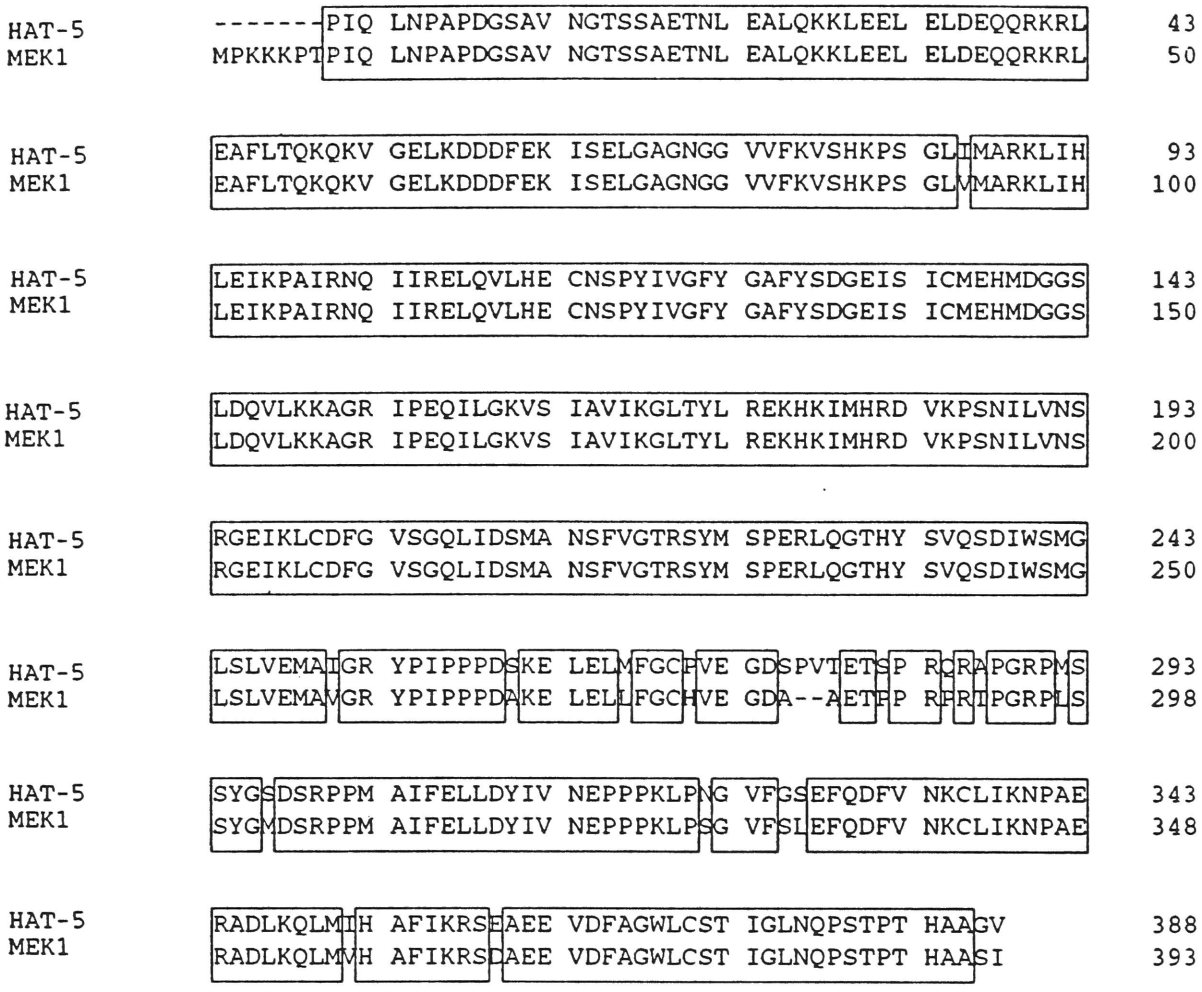
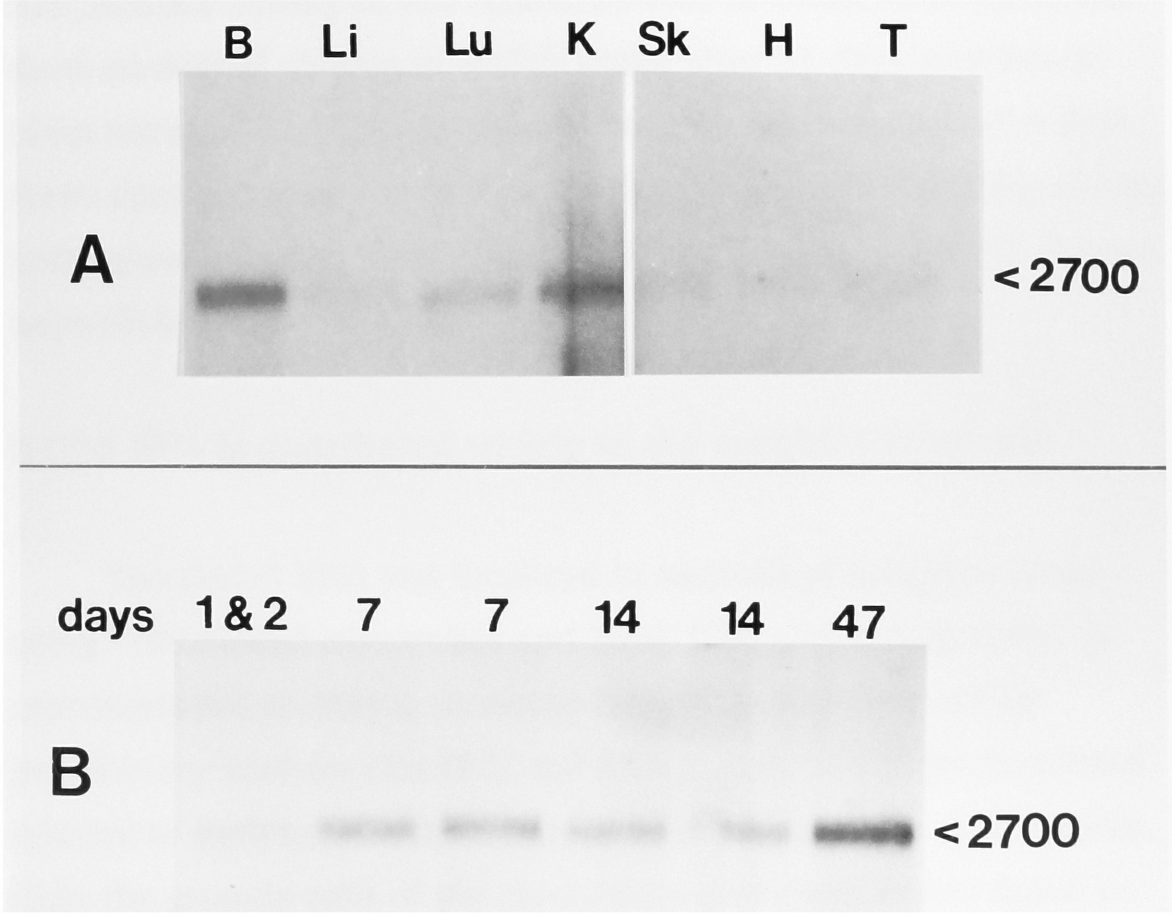

HAT-5 -------PIQ LNPAPDGSAV NGTSSAETNL EALQKKLEEL ELDEQQRKRL 43
MEK1 MPKKKPTPIQ LNPAPDGSAV NGTSSAETNL EALQKKLEEL ELDEQQRKRL 50
HAT-5 EAFLTQKQKV GELKDDDFEK ISELGAGNGG VVFKVSHKPS GLIMARKLIH 93
MEK1 EAFLTQKQKV GELKDDDFEK ISELGAGNGG VVFKVSHKPS GLVMARKLIH 100
HAT-5 LEIKPAIRNQ IIRELQVLHE CNSPYIVGFY GAFYSDGEIS ICMEHMDGGS 143
MEK1 LEIKPAIRNQ IIRELQVLHE CNSPYIVGFY GAFYSDGEIS ICMEHMDGGS 150
HAT-5 LDQVLKKAGR IPEQILGKVS IAVIKGLTYL REKHKIMHRD VKPSNILVNS 193
MEK1 LDQVLKKAGR IPEQILGKVS IAVIKGLTYL REKHKIMHRD VKPSNILVNS 200
HAT-5 RGEIKLCDFG VSGQLIDSMA NSFVGTRSYM SPERLQGTHY SVQSDIWSMG 243
MEK1 RGEIKLCDFG VSGQLIDSMA NSFVGTRSYM SPERLQGTHY SVQSDIWSMG 250
HAT-5 LSLVEMAIGR YPIPPPDSKE LELMFGCPVE GDSPVTETSP RQRAPGRPMS 293
MEK1 LSLVEMAMGR YPIPPPDAKE LELIFGCHVE GDA--AETPP RPRTPGRPLS 298
HAT-5 SYGSDSRPPM AIFELLDYIV NEPPPKLPNG VFGSEFQDFV NKCLIKNPAE 343
MEK1 SYGMDSRPPM AIFELLDYIV NEPPPKLPSG VFSLEFQDFV NKCLIKNPAE 348
HAT-5 RADLKQLMIH AFIKRSEAEE VDFAGWLCST IGLNQPSTPT HAAGV 388
MEK1 RADLKQLMVH AFIKRSDAEE VDFAGWLCST IGLNQPSTPT HAASI 393

FIGURE 10. Northern analysis of HAT-5 mRNA in canary tissues and developing zebra finch forebrain. **Panel A:** ^{32}P-labelled cDNA probe from the HAT-5 clone hybridizes to a dominant band of ~2700 bp which is most abundant in brain and kidney, but also detected in all other tissues examined. Tissues of RNA origin (l. to r.) include forebrain, liver, lung, kidney, skeletal muscle, heart, and testes. **Panel B:** Developmental profile of HAT-5 RNA expression. Each lane represents poly(A)+ RNA isolated from the forebrain of a single different zebra finch of the indicated age, except for the 1-2 day sample, in which a 1-day and a 2-day sample were pooled.


B
Li
Lu
K
Sk
H
T
A
<2700
days
1&2
7
7
14
14
47
B
<2700

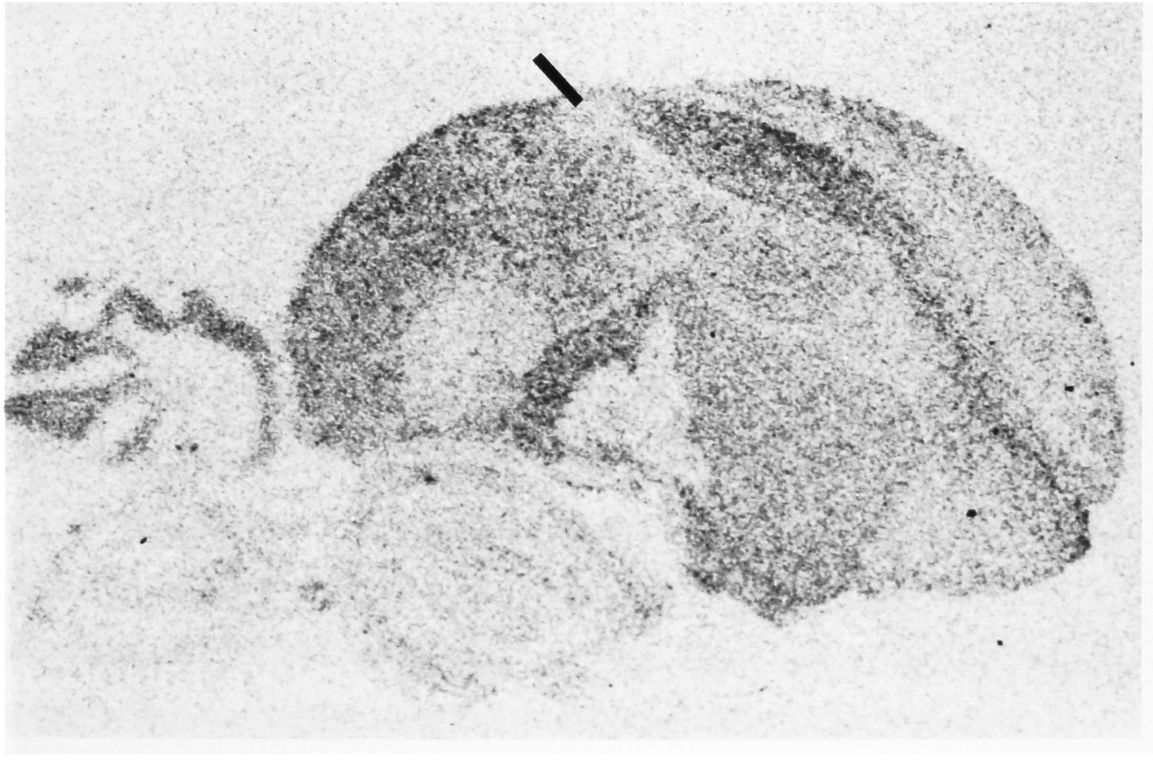
Barely detectable levels of expression were observed for heart, testes, skeletal muscle, and liver.

HAT-5 expression is very low in the zebra finch forebrain at 1-2 days post-hatch (lower panel), but RNA levels rise significantly by day 7 and remain stable through day 14. By day 47, the level of HAT-5 RNA has again increased to a level representative of adults; comparisons among *in situ* hybridizations of zebra finch forebrain show no overall change in HAT-5 signal after 35 days post-hatch (data not shown). Hence, the most striking increase in HAT-5 RNA levels occurs during the first week post-hatch, a period of significant brain growth during which brain mass nearly quadruples (J. George, unpublished observation).

HAT-5 RNA is distributed widely in the songbird forebrain

The HAT-5 RNA was localized in sections of songbird brain using ^{35}S-labelled riboprobes and X-ray film autoradiography. A representative section is shown in Figure 11 As predicted by preliminary analysis (Table I), the RNA is enriched in the forebrain relative to such non-forebrain areas as brainstem and optic tectum. Only the granule cells of the cerebellum give comparable signal to forebrain. Labelling is heterogeneous within the forebrain, with relatively low levels of signal in song nucleus HVC and in the archistriatum.

FIGURE 11. Distribution of HAT-5 mRNA in sagittal sections of adult male canary brain. Signal detected by in situ hybridization with 35S-labelled riboprobes. This section represents a Spring male canary. Song nucleus HVC is indicated with a line segment.



Discussion

The sequence of canary HAT-5 suggests that it may play an important role in signal transduction events in the songbird forebrain, possibly in a pathway transducing growth factor and other receptor-mediated signals. Comparison to a newly reported murine sequence (Crews, et al., 1992) strongly suggests that HAT-5 is the homolog of a protein known as MEK1 (for MAP-kinase or ERK kinase).

MEK1 was initially characterized as an activity regulating MAP (mitogen-activated protein) kinases, also known as ERKs (extracellular signal-regulated protein kinases), which are activated in response to mitogenic stimuli including epidermal growth factor (EGF), nerve growth factor (NGF), platelet-derived growth factor (PDGF), and insulin (Cooper, et al., 1984), (Cobb, et al., 1991a). MAP kinase may also be stimulated by protein kinase C acting through an independent mechanism (deVries-Smits, 1992; Gupta, 1992; Gallego, 1992). In either case, activation of MAP kinase requires both threonine and tyrosine phosphorylation (Anderson, et al., 1990). It has been demonstrated that N-ras-induced differentiation of PC12 cells can be inhibited by disrupting MAP kinase activity (Qiu and Green, 1992). Thus MAP kinase is implicated in several growth factor signal transduction pathways and in at least one pathway leading to neurite outgrowth.

Study of the upstream events mediating MAP kinase activation have led to the partial purification of MEK1 from rabbit skeletal muscle (Wu, et al., 1992a), Xenopus oocytes (Kosako, et al., 1992),

and ultimately to the cloning of its cDNA in mouse (Crews, et al., 1992). Purified MEK1 from Xenopus oocytes has both serine/threonine and tyrosine kinase activities (Kosako, et al., 1992), making it unique among known protein kinases. This broad specificity may account for its ability to stimulate MAP kinase activation *in vitro* (Nakielny, et al., 1992), despite the requirement of both threonine and tyrosine phosphorylation for MAP kinase activation.

The peptide predicted by HAT-5 was compared to that encoded by the murine MAP kinase activator cDNA, denoted MEK1. The precise start site for the HAT-5 peptide cannot be determined, as the HAT-5 open reading frame extends to the extreme 5' end of the cDNA, and additional residues, including a starting methionine, are almost certainly encoded by 5' sequences not present in this clone. The HAT-5 cDNA encodes all but 7 amino-terminal residues present in MEK1; the sequences are otherwise 95% identical.

Hence, canary HAT-5 seems to represent the canary homolog of a protein which has been linked to signalling of mitogenesis and neurite outgrowth. It is interesting that the RNA is most abundant in brain (see Fig. 10, also Crews, et al., 1992), suggesting it probably plays an role in the adult nervous system, as well as in development. An obvious question is whether HAT-5 is involved in adult neurogenesis, a well-characterized phenomenon in the avian brain. Some preliminary data have suggested an enrichment of HAT-5 RNA in neurogenic ventricular zone of adult zebra finches, relative to underlying telencephalon (where it is also detected). Further experiments must determine whether similar enrichment is also

observed in the non-neurogenic fourth ventricle. Efforts are underway to generate antibodies to HAT-5 to further confirm its localization in the songbird brain and to determine its intracellular distribution.

Chapter 5. A song circuit-enriched mRNA, HAT-14, encodes a putative PKaseC-regulated calmodulin-binding protein

HAT-14 encodes a novel protein with homology to GAP-43 and RC3/neurogranin

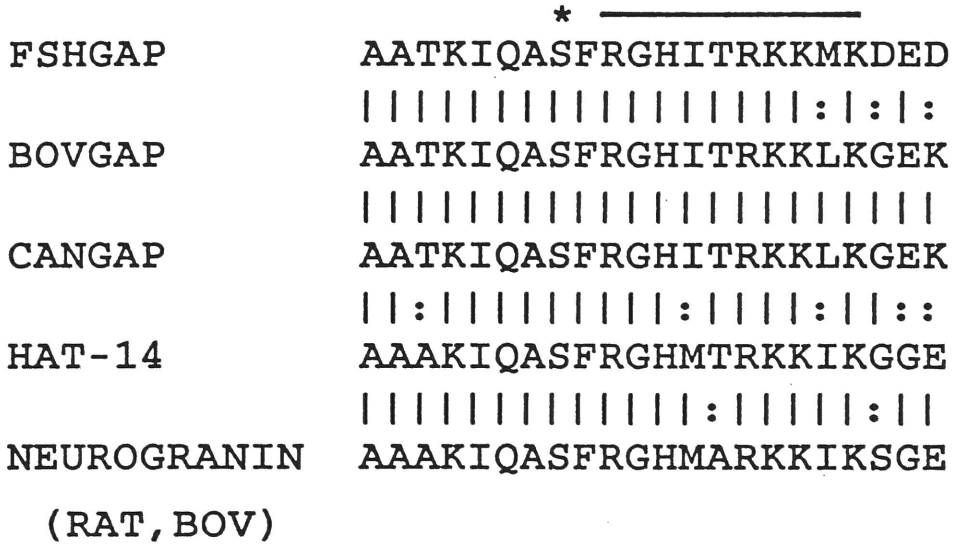
HAT-14 RNA is ≥8-fold enriched in the HAT relative to non-forebrain, and is present at a relative abundance of $3x10^{-4}$. When the 2341 bp HAT-14 insert is used to probe Northern blots of poly(A)$^+$ RNA from canary or zebra finch brain, a dominant species of ~1000 bp is detected (see Table 1), indicating that the cloned insert contains sequences not present in the HAT-14 RNA, an issue which will be addressed later in this section.

The nucleic acid sequence of the HAT-14 cDNA insert is shown in Figure 12. Analysis of possible open reading frames suggests a protein of 73 amino acids which includes a 22 residue domain common to the neural growth associated protein, GAP-43, although the two sequences are otherwise unrelated. The canary GAP-43 homolog has been cloned and sequenced (Clayton et al., unpublished data), and it likewise predicts a protein which resembles HAT-14 only within this 22 amino acid stretch. An alignment of HAT-14 with GAP-43 of several species is shown in Figure 13. Perhaps significantly, this domain has been characterized in GAP-43 and shown to include a site for calmodulin binding (Alexander, et al., 1988), as well as a conserved serine thought to be the principal

FIGURE 12. Nucleotide sequence and minimal translated product of HAT-14 cDNA. The 2341 bp HAT-14 cDNA insert. Double arrows indicate the border between sequences which hybridize to the 1000 bp HAT-14 RNA and upstream sequences which do not. An open reading frame with partial identity to sequences in GAP43 and another protein, RC3/neurogranin, are boxed. Peptide sequences indicated by dashed (----) lines were synthesized and used to immunize mice for production of HAT-14 antisera.

```
TTTTTTTGGTTTACAGAACAGGTTGTGTTTACTTCAGTTCACAAAGTAAAGCCACCAGCT    60
TTAATTCTCCATAAAAATAAAACAATAGGGCAAGTGCATGATTCAGAGGGCTTGGTGCAA   120
ATTTTTGCAAAGTAACAGATACAAAACCAAAATAATTAAAATCATACATATGTGTAAAAC   180
AAAAAAAAATTCAAGTTATAACCAGGACTGATTCCTATTAGCCCTAGAGATGATCCAAGA   240
ATATGTCAGGAAATAAATACATTGTTCTAGTTTTCACCATAAAAGAGGGTGCTGCTCTCT   300
AATCGAAATGGTTGGGTATTTACAGATGGCAAATGGGTTACAGGAGAAACAGAAAGAAGA   360
AAAGCATTCATAAGAGTGAACTGAAATGCTGCAGGGTGACCACTTCCCCACACACCTCGG   420
TGTGACTAAGAGTACACTAAGTCACAGCAAGGGCTGCAGAAATTAACTGTGAGCAGGACA   480
TCATCACCTGCCCCAGGCTGGAGGGACTGTGCCAAGGTGAGACCAGCATGGTGTGGCTGC   540
CACCACAGAGATTCTGGAGCTCCACCACAATCCATGGCTGCCCTGGGGCAGATGGCAGTA   600
AGTTGGCCTGGTCAGTCCAATTTATGAACAATTTATCCTACAACCCACCTGAAGGGAATG   660
CTGCTTCAAGGCACTTCCTTCCTGGCCCTGCTGGACTGGGGGTGCTCTGAGGAAGAGGAG   720
GCAGCCAGCACCACTGAACCTCTTCTCCTGACATGGGAAGCACAGCTGGTGATGTGATCA   780
CTCAGCCTTTCCCCAAAGGCCAAGAGCTCCAGTGGCAGGAGGGAAAAAAAGATTCTTCCA   840
AGTATCTGTCTCAACATTGCAAAGCACAGCAGACAAAGCAACACCCTCATTTATCAAGGC   900
AGCATGCCTTGTGCTCATAGCTGGTATTTCTGATGCTTTGTTGCCATATAATAACCAGCT   960
CTGCTTTTTCTGCATGTGCTACTAATGCCAAATCCTGGCAGGTCAGAGTTGTGCTCCAGT  1020
GCAGACAGTGGAGCAGATGTTTAATGCCCAACAGAAGCAGGGGAAATTCTGCAGTTAATG  1080
TCATAGCATGCAAAATAACATCCCAAAGGCTCATCCTAACCTTCTGTTTCCTTATCAAGA  1140
CACGCTTATTTACCAAGTATCACTTGGAGTAGATTCAAGTGGTAGTTGAGTTGTGTCCAT  1200
ATTTTCAATGGGATTTGACATTTAAGGCAGCACTGCAGTTCACCTCTAAGCCTATAACAA  1260
CACTTGCAAATCTGCTTAGCAAGACACCAGCAGAGTATCCCAGGGACAGCTCAAACAGAT  1320
TGGCTCTGCCCCCACAGAAGCAGCACTGGAGTACCAAGAGATGCTACAAAGCAGACACTG  1380
CAGACTGCAGTGCCTGACACAGACACCTGCAAACTCACCCACTCCCAATCCTCTCAATAA  1440
AAATAAAACCAAAGACTGCAAAGAGCTCTCACTTTATCTTAAAAGCAATGCTGCACTGAG  1500
TCAGATACCACTGGAGGTCAGGAGCACTTCATGAATACACTGAGCCAGGATTCCTTTCCA  1560
CACCCCGCTGATTATCCTTGGTCTCATGAAATCCACAAGTGCTAAAAGTACCAGGGTAGG  1620
CTCAGGATGAAATACTGATCATCACCTCCTGATCTGGAGTCACAATGGCCCAGTGCTGAG  1680
AAGCACTTTTACATCTCCTGCCCATCTGATTTTCTCTTTCATCATAGTCCTATCGCTACT  1740
GATATTTTGAGAGCAAATGGATGCCAAATGCCAGGATGCTCGGTACATTTTTCACCCAAT  1800
TTACACACCCCAACCATCCTCTGCACCCAATTTATAGCAGAACCCCCAAGACATGGCTGG  1860
AACTCCGGAGCCCCTCGCCCGCCGCTGGCCGCCCACCCGCCCTCCGCCATGGACTGCTGC  1920
                                                  M  D  C  C

AATGAGGGAGCCTGCACAAAGCTGGACGAGGACATCCTGGACATCCCTTTGGACGATCCC  1980
N  E  G  A  C  T  K  L  D  E  D  I  L  D  I  P  L  D  D  P

GATGCCAACGCGGCAGCTGCCAAGATCCAGGCTAGTTTCCGTGGCCATATGACCCGCAAG  2040
D  A  N  A  A  A  A  K  I  Q  A  S  F  R  G  H  M  T  R  K

AAGATCAAAGGGGGTGAGATTGATCGGAAAACCAAGGACGCCGAGTGCGCCAACAGCACC  2100
K  I  K  G  G  E  I  D  R  K  T  K  D  A  E  C  A  N  S  T

CGCGGCGGCGACCTCCGCAACGGCGACTAGGGGCCACCAGCTGCTCACCGGAAACCCCTC  2160
R  G  G  D  L  R  N  G  D

GCCACCGACTCCCTCCCACCGCCGCCCGCCGAGGCCCAGGAGCCCCTGCTTCACCCCCTG  2220
CCCCTCGCATGCATCCCCCGGGGTTCCTCCACAGCCAGGCCCTGCCCCTGTCCCTGGCTG  2280
CCCCTCCCCACCCCAGACACCCCCTGCCCCAATAAACGCCCCTGCCAGAGCCAAAAAAAA  2340
A                                                              2341
```

FIGURE 13. Alignment of a 22 amino acid domain conserved in GAP43, HAT-14, and RC3/neurogranin. The conserved serine (*) is a target for protein kinase C phosphorylation, while the overlined sequence has been demonstrated to mediate calmodulin binding.


*
FSHGAP AATKIQASFRGHITRKKMKDED
BOVGAP AATKIQASFRGHITRKKLKGEK
CANGAP AATKIQASFRGHITRKKLKGEK
HAT-14 AAAKIQASFRGHMTRKKIKGGE
NEUROGRANIN AAAKIQASFRGHMARKKIKSGE
(RAT,BOV)

target of protein kinase C phosphorylation (LaBate and Skene, 1989)(Apel, et al., 1990).

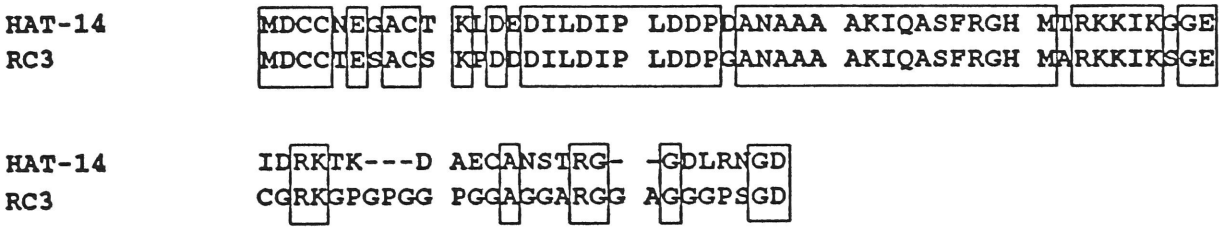
Recently, a third sequence sharing this domain has been described. Known as RC3 (rat)(Watson, et al., 1990) or neurogranin (bovine)(Baudier, et al., 1991a), it is otherwise distinct from GAP-43. An alignment of RC3 with HAT-14 is shown in Figure 14. While the two sequences are closely related over the first 50 amino acids, they diverge sharply at their carboxyl termini.

With this suggestive sequence data, it became essential to establish whether this particular open reading frame in fact corresponds to the 1000 bp RNA detected by the HAT-14 cDNA. Probes representing small fragments of the HAT-14 insert were used to probe Northern blots of poly(A)$^+$ RNA. Probes representing sequences downstream of nucleotide 1865 (indicated in Fig. 12) detect the 1000 bp HAT-14 band, while probes representing upstream sequences give no signal (data not shown). Upstream sequence may represent a very rare transcript which is undetectable by Northern analysis. This is supported by the presence of poly(T) sequence at the 5' end of the clone, which could represent the poly(A) tail of a clone fused head-to-head with HAT-14 in the opposite orientation.

Antipeptide antibodies detect an 18kD species in extracts of zebra finch brain

For further assurance that this encoded protein is actually produced, antibodies were prepared against two distinct peptides predicted by the HAT-14 sequence (Fig. 12,

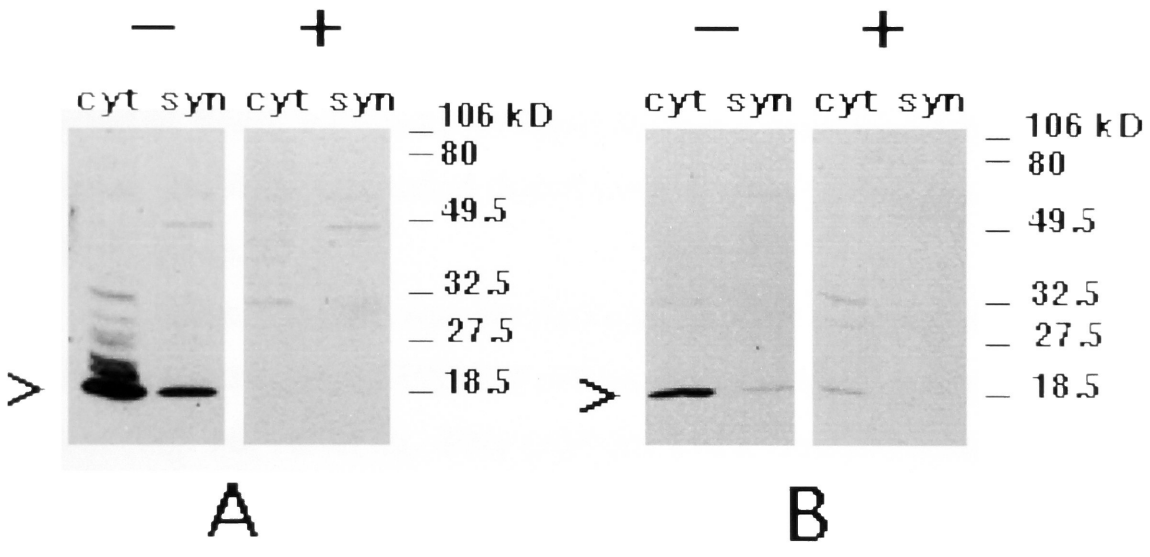
FIGURE 14. Alignment of canary HAT-14 protein and rat RC3. The open reading frame containing the conserved 22 amino acid domain encodes a protein with a minimum size of 73 amino acids. This sequence is shown aligned with rat RC3. Conserved residues are boxed. The sequences are 84% identical over the first 50 amino acids; thereafter, similarity drops to below 50%.


HAT-14 MDCCNEGACT KLDEDILDIP LDDPDANAAA AKIQASFRGH MTRKKIKGGE
RC3 MDCCIESACS KPDDDILDIP LDDPGANAAA AKIQASFRGH MARKKIKSGE
HAT-14 IDRKTK---D AECANSTRG- -GDLRNGD
RC3 CGRKGPGPGG PGGAGGARGG AGGGPSGD

dashed lines). The first peptide corresponds to internal residues 9-25, a sequence which is well conserved between HAT-14 and RC3, but does not overlap with the domain of GAP-43 homology. A second peptide was synthesized representing residues 60-73, which are the 14 carboxy-terminal residues of the presumed HAT-14 protein. These residues are not well-conserved between HAT-14 and RC3.

The peptides were conjugated to the carrier protein KLH and used to immunize mice. Sera were screened against the appropriate peptide conjugated to BSA (so as not to detect antibodies reactive with KLH), and polyclonal ascites were generated in positively reactive mice. Antibodies were used to probe crudely fractionated extracts of zebra finch brain on Western blots followed by chemiluminescent detection (ECL, Amersham). Representative blots are shown in Fig. 15. Panels A and B represent antibodies raised against the internal and carboxy-terminal sequences, referred to as H14-i and H14-c, respectively. Both antibodies detect a dominant band at 18kD, and in each case the signal can be preabsorbed by incubation with the immunizing peptide. Antisera to the internal peptide detect 5 additional bands between 20 and 30 kD which are also specific by these criteria. The target proteins are likely soluble, as they are enriched in the cytosolic fraction relative to the synaptosomal fraction (a crude preparation of synaptosomes, the membranous vesicles which form when synaptic terminals are disrupted by homogenization; synaptosomes are composed of synaptic membrane and associated synaptic proteins, and may enclose synaptic vesicles and other cytosolic elements.)

FIGURE 15. Western analysis of HAT-14 immunoreactivity in crudely fractioned extracts of zebra finch brain. **Panel A:** a polyclonal ascites against the HAT-14 internal synthetic peptide is used to probe extracts of zebra finch brain. An arrow indicates the dominant band recognized by this antibody. (+): immunoreactivity was specifically absorbed from the probe be preincubation with its target peptide; (-) immunoreactivity was not affected by preincubation with an unrelated peptide. Crude cytosolic and synaptosomal fractions are labelled. **Panel B:** a polyclonal ascites against HAT-14 C-terminal residues is used to probe a blot identical to that in Panel A. (-): A band of similar size as that detected in Panel A is indicated by an arrow. (+): This reactivity is also preabsorbed by incubation with its specific peptide. Positions of molecular weight standards which were visualized in the gel are indicated, along with their corresponding sizes.


−
+
cyt syn cyt syn
106 kD
80
49.5
32.5
27.5
18.5
A
−
+
cyt syn cyt syn
106 kD
80
49.5
32.5
27.5
18.5
B

Northern blot analysis indicates that HAT-14 is brain-specific and developmentally regulated in the forebrain

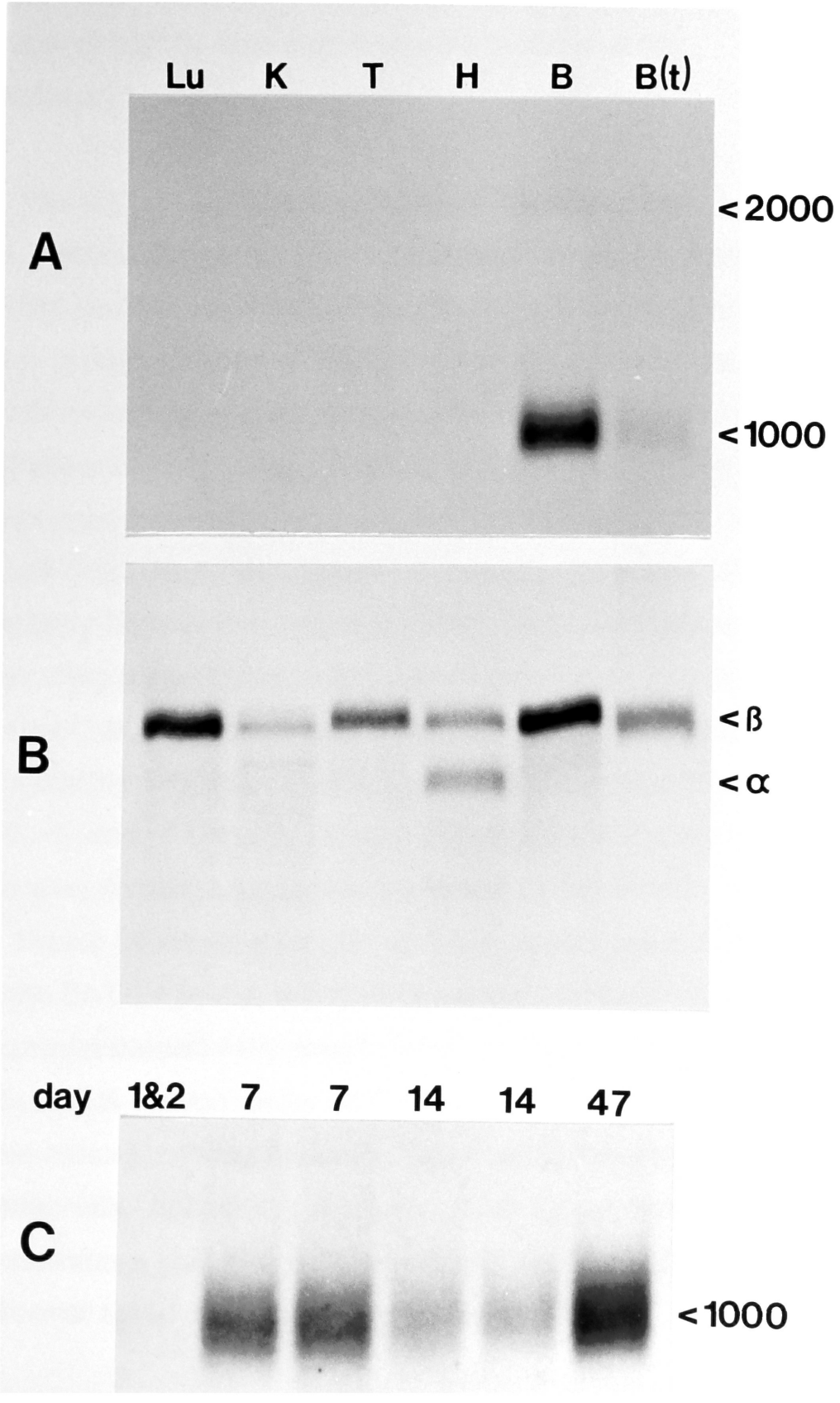
The HAT-14 cDNA was used to probe Northern blots of poly(A)+ RNA representing a variety of canary tissues including lung, kidney, testes, heart, and forebrain, as well as forebrain total cytoplasmic RNA (Fig. 16, Panel A). The 1000 bp HAT-14 transcript was detected only in the brain samples. An additional minor band was observed in the forebrain poly(A)+ sample at approximately 2000 bp. When identical blots were probed for actin, the ubiquitous β-actin transcript was detected in all tissues, and α-actin was detected in heart, as would be expected for a muscular tissue (Fig. 16, Panel B). It is concluded therefore that HAT-14 mRNA is specific to brain tissue.

Northern analysis was similarly applied to determine the developmental onset of HAT-14 mRNA expression in young zebra finches (Fig. 16, Panel C). Poly (A)+ RNA was isolated from the forebrains of zebra finches of known age, blotted, and probed with the HAT-14 cDNA. The signal detected for each age was quite different. HAT-14 RNA is not detectable at 1-2 days post-hatch. By 7 days, appreciable signal is detected, although it appears to be less than that observed by 47 days. Surprisingly, the signal detected at 14 days is lower than that observed at 7 days. These data suggest that HAT-14 is rather dynamically regulated during development.

FIGURE 16. Northern analysis of HAT-14 mRNA in canary tissues and developing zebra finch forebrain.

Panel A: ^{32}P-labelled cDNA probe from the HAT-14 clone hybridizes to a dominant band of ~1000 b which is detected only in brain; a minor transcript of ~2000 b is also indicated. Tissues of RNA origin (l. to r.) include canary lung, kidney, testes, heart and forebrain (1 microgram polyA+ RNA each). The lane on the far right contains 15 micrograms of total cytoplasmic RNA from zebra finch brain. **Panel B:** an identical blot probed for actin; the beta-actin transcript is observed in all tissues, whereas the alpha isoform is detected in heart as would be expected for muscle.

Panel C: Developmental profile of HAT-14 RNA expression. Each lane represents 1 microgram poly(A)+ RNA isolated from the forebrain of a single different zebra finch of the indicated age, except for the 1-2 day sample, in which a 1-day and a 2-day sample were pooled.


Lu
K
T
H
B
B(t)
A
< 2000
< 1000
B
< β
< α
day
1&2
7
7
14
14
47
C
< 1000

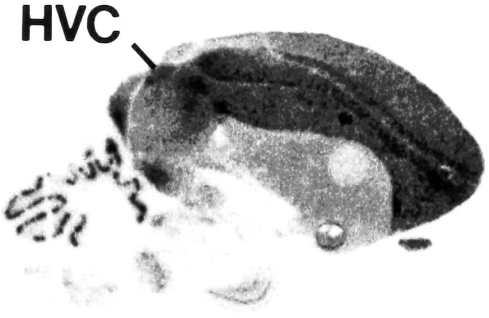
HAT-14 mRNA is differentially regulated in the avian song circuit and highly enriched in Purkinje cells of the cerebellum

The HAT-14 RNA was localized in the forebrain using *in situ* hybridization. Figure 17 shows sections from an adult male canary in Spring and an adult male zebra finch, both representative of periods of high song production and low song plasticity. The hybridization patterns are very similar for the two species, with signal generally high in the forebrain, much lower in the midbrain and optic tectum, and low in the cerebellum except for the cerebellar Purkinje cells, where the signal is very high. HAT-14 RNA is moderately high in the song nuclei HVC and lateral MAN relative to surrounding neostriatum, and noticeably lower in Area X relative to the rest of LPO. As described in Chapter 3, Figure 5, control probes do not distinguish these nuclei from adjacent tissues, and while HAT-2, -5, and -3 (Figs. 5, 11, and 26) are all differentially regulated in the song circuit, each is present in a unique pattern.

Figure 18 shows a section of canary tissue hybridized with the HAT-14 probe, exposed to autoradiographic emulsion, and counterstained with cresyl violet. In Panel A, the cerebellum is viewed under dark field to demonstrate the intense concentration of silver grains over the single layer of Purkinje cells. In Panel B, a higher power bright field image demonstrates a ring-like, cytoplasmic distribution of silver grains over these very large neurons, with much

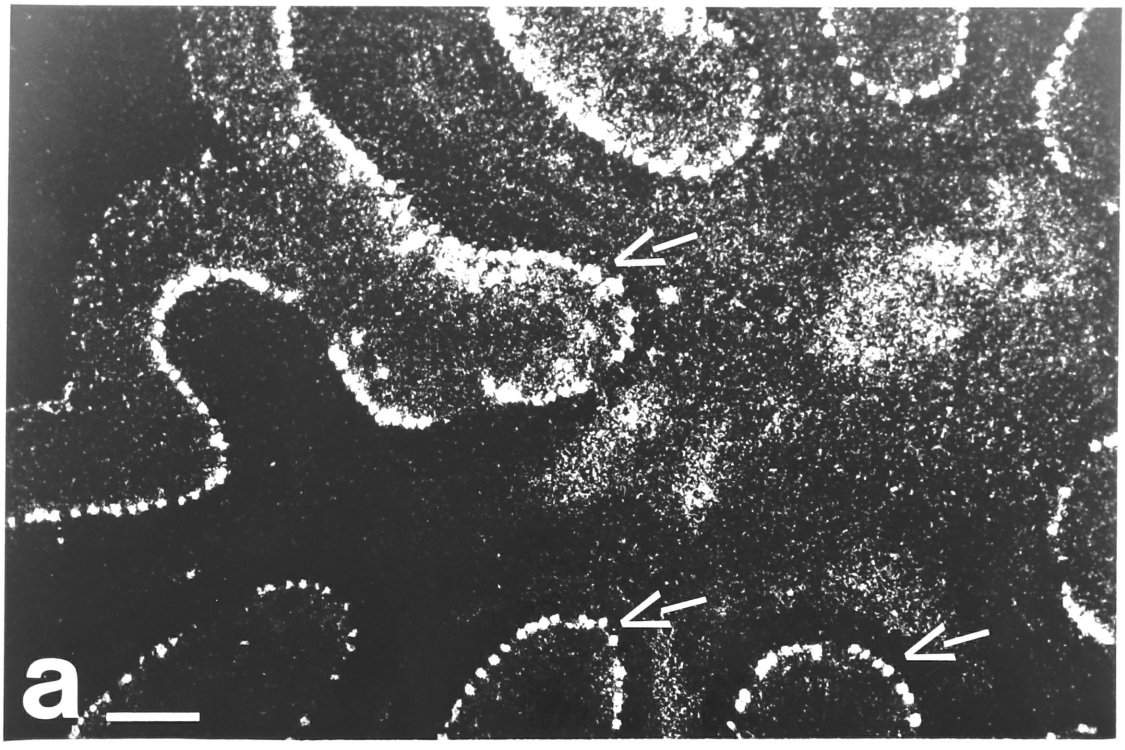
FIGURE 17. Distribution of HAT-14 mRNA in sagittal sections of adult male canary and zebra finch brain. **Upper panel:** a sagittal section from a male Spring canary. The position of song nucleus HVC is indicated. Also distinguishable but unlabelled is the song nucleus Area X, a circular region of relatively low staining in the anterior telencephalon (refer to Figure 1). The single layer of cerebellar Purkinje cells is also evident. The dark area in archistriatum is distinct from nucleus RA (as revealed by inspection of counterstained material); RA expresses this sequence at levels equivalent to the surrounding archistriatum. **Lower panel:** a sagittal section from a male zebra finch. Note the similarity in patterns of HAT-14 expression between these related species.

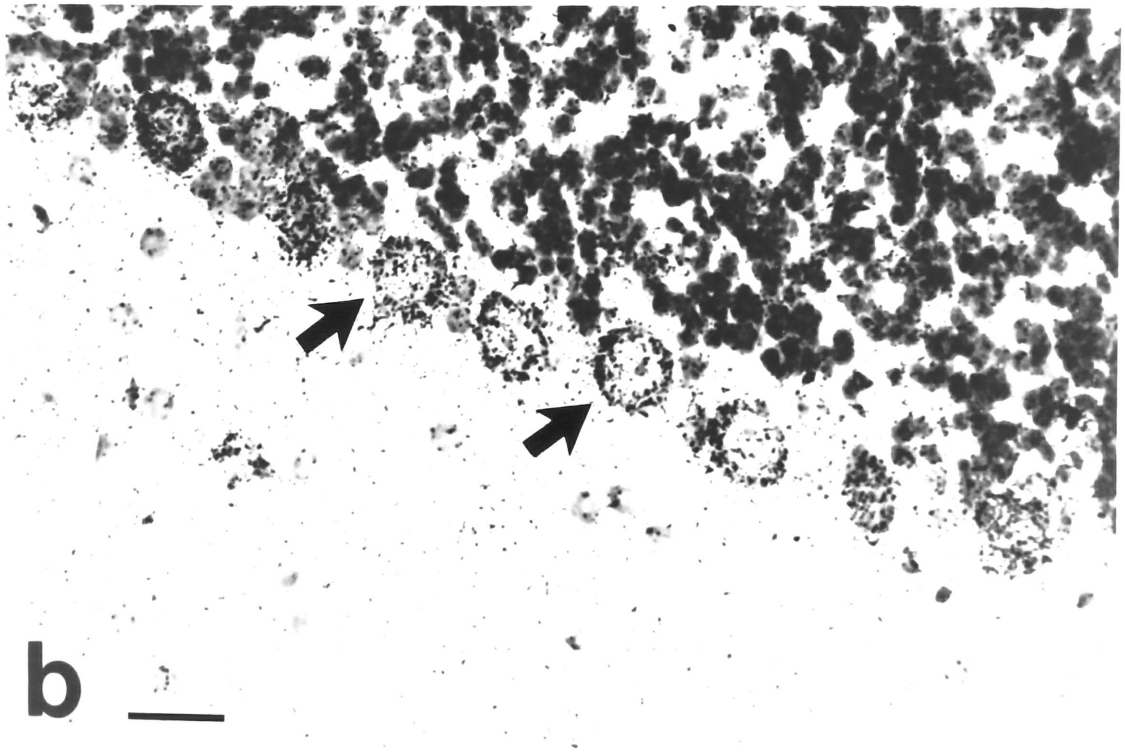
CANARY



ZEBRA FINCH

FIGURE 18. Expression of HAT-14 mRNA in Purkinje cells of the cerebellum (dark field and bright field autoradiographs). Hybridized sections canary brain were exposed to autoradiographic emulsion, developed, and stained for Nissl substance. **Panel a:** under dark field, a concentration of silver grains is seen over the single layer of Purkinje cell neurons; size bar = 200 μm. **Panel b:** under bright field, layers of the cerebellum are clearly distinguished. The single layer of Purkinje cells is indicated by dark arrows. Above this layer are the densely packed cells of the granule layer; below is the molecular layer, which is much less cell-dense; size bar = 20 μm


a


b

fewer grains over the adjacent granule cell and molecular layers.

Immunocytochemistry localizes HAT-14 protein within forebrain neurons and cerebellar Purkinje cells

To determine more precisely the intracellular distribution of the HAT-14 protein, and to investigate which cell types in the forebrain express the protein, immunocytochemistry was applied to perfusion-fixed, PEG embedded zebra finch brain tissue. Figures 19 and 20 show tissue stained with polyclonal ascites H14-c (reactive with the HAT-14 C-terminal peptide), followed by fluorescein-conjugated goat anti-mouse secondary antibody, and detected by fluorescence microscopy.

In Figure 19, a near-vertical row of brightly stained Purkinje cell bodies is evident. These large neurons have vast dendritic arborizations in the molecular layer of the cerebellum. Their dendrites, extending from the Purkinje cell bodies into the molecular layer on the right side of the field, are clearly labelled with antibodies to HAT-14.

Figure 20 shows immunocytochemical staining of a field within zebra finch neostriatum, demonstrated by *in situ* hybridization to express high levels of HAT-14 mRNA. Panels a and c depict similar fields stained, respectively, with mouse polyclonal ascites against the HAT-14 C-terminal peptide or with anti-mouse secondary only. Panels b and d show counterstaining of these same fields with a stain for cell nuclei. HAT-14 is present predominantly in cells with large,

FIGURE 19. Immunocytochemical localization of HAT-14 in cerebellar Purkinje cells. Immunofluorescent staining with H14-i reveals a dense arborization of Purkinje cell dendrites (arrow) size bar=10 μm

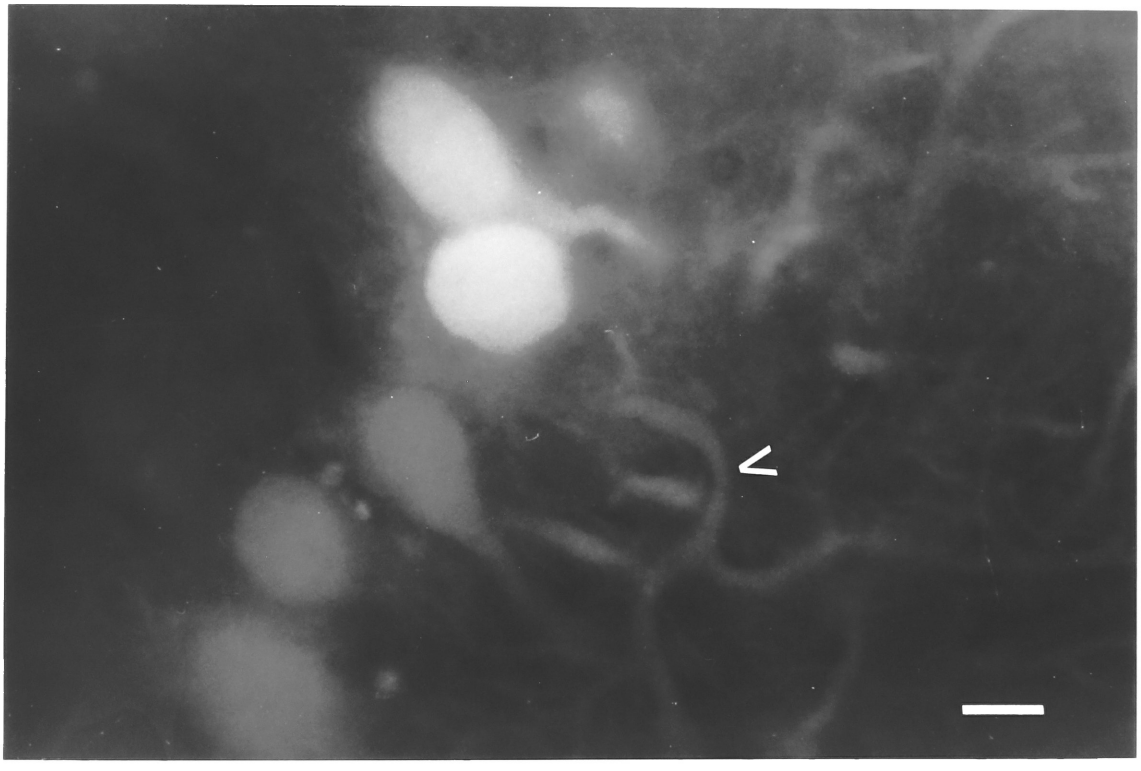
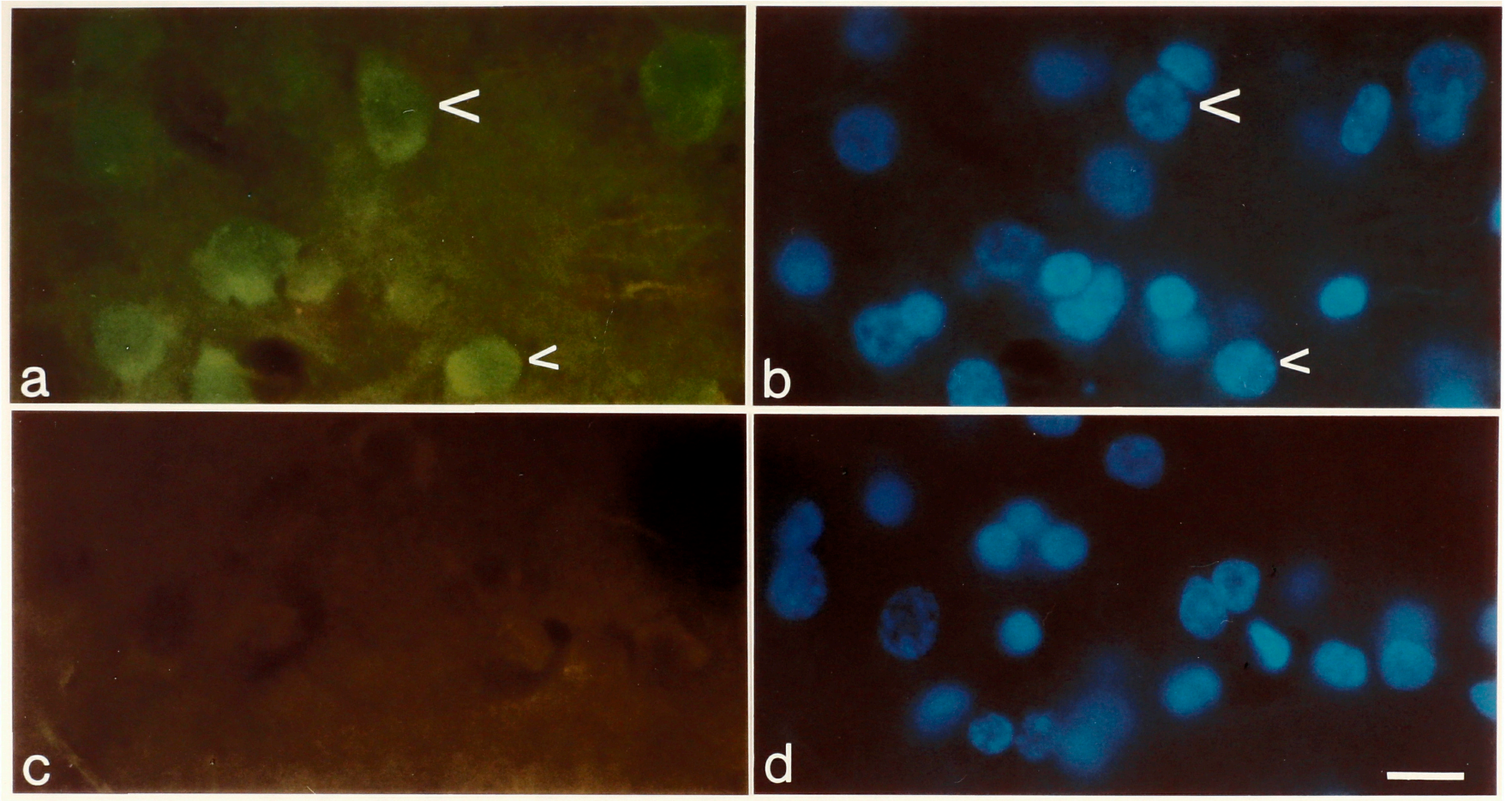


FIGURE 20. Immunocytochemical localization of HAT-14 in neurons of the neostriatum. **Panel a:** A field of cells in the anterior neostriatum is immunofluorescently stained for with H14-i. Panel c: A similar field in an adjacent section stained with secondary antibody only. **Panels b and c:** fluorescent counterstain for cell nuclei is detected in another fluorescence channel. Corresponding cells in side-by-side panels are indicated by identical arrows. size bar= 10 μm


a
b
c
d

pale-staining nuclei characteristic of neurons. Immunostaining of several neuronal processes is also evident. A low level of fluorescence observed in the control is attributed to autofluorescence by the tissue, and is visibly different in color from the fluorescein fluorescence. Autofluorescence was particularly pronounced in the song nuclei HVC and RA(data not shown), and immunocytochemical detection of HAT-14 in these areas will require a non-fluorescent detection method.

Discussion

HAT-14 is a novel, brain-specific RNA which is differentially regulated in the song circuit of canaries and zebra finches. Northern blot analysis indicates that the RNA is regulated dramatically in the forebrain during early development of the zebra finch, with a large increase in expression during the first week post-hatch. As songbirds are altricial animals, this corresponds with a period of significant brain growth and synaptogenesis (Herrmann and Bischof, 1986). Levels of RNA drop somewhat during the second week, before rising to the high level observed at 47 days of age. This level approximates that seen in the adult, as overall expression in the forebrain does not seem to vary beyond day 35 (data not shown), although regulation in small subregions of the forebrain would not be detected by this method.

In situ hybridization indicates that the RNA is distributed rather broadly in the forebrain, with rather lower levels in the the midbrain, optic tectum, and cerebellum, consistent with the forebrain

enrichment reported in Table 1. However, very high levels of HAT-14 RNA are observed in a restricted population of cells within the cerebellum, the cerebellar Purkinje cells, which are situated in a single layer between the granule cell and molecular layers of this structure. The Purkinje cells are clearly visible on sheet film autoradiograms of both canary and zebra finch brain sections (Fig. 17). Emulsion autoradiography was used to confirm the localization of HAT-14 RNA to these cells. Signal was distributed around the perimeter of the Purkinje cell nucleus, as would be expected for a cytoplasmic RNA (Fig. 18).

Within the forebrain, HAT-14 RNA is regulated rather strikingly within the song circuit of adult male canaries in Spring and adult male zebra finches, both representative of periods of high song production and low song plasticity (Nottebohm, et al., 1986), (Marler, et al., 1988). HAT-14 is the only HAT RNA to show enrichment in HVC relative to surrounding neostriatum. HAT-14 is also somewhat high in LMAN relative to surrounding neostriatum, and rather low in Area X relative to the rest of LPO (Figure 1 indicates the positions of these nuclei). One hypothesis still being explored is that HAT-14 RNA may be dynamically regulated in the song circuit during periods of varying plasticity. When male canaries from Spring (March-May) and Fall (October) were hybridized for HAT-14 RNA, no obvious differences in HAT-14 expression were observed, although in contrast to Spring, Fall is characterized by high song plasticity (Nottebohm, et al., 1986). However, changes in HAT-14 expression might be so transient as to have been missed in this small sample of birds (4 Fall birds, 7 Spring birds), and a more extended time course

would be required to exclude changes occurring at other times of the year. Preliminary evidence in the zebra finch, however, suggests that HAT-14 levels may increase within song nucleus HVC during the critical period for song development. Confirmation and quantification of this result is still ongoing, but it may suggest that HAT-14 has some stabilizing effect in the motor pathway for song production as the behavior develops and matures

The protein encoded by HAT-14 is similar to a mammalian protein, RC3/neurogranin, but is distinct in several respects. While the sequences are highly conserved over their N-terminal 50 amino acids, they diverge sharply at their C-termini. A glycine-rich sequence at the C-terminus of RC3 resembles a motif found in extended collagen chains, leading others to speculate that it may form multimeric helices with itself or other proteins, resulting in multimeric protein complexes (Watson, et al., 1990). This motif is not represented in the HAT-14 sequence. Furthermore, cellular level analysis of the distribution of RC3 RNA indicates that it is specifically absent from all layers of the rat cerebellum (Watson, et al., 1990). Likewise, no neurogranin immunoreactivity was detected in any layer of the rat cerebellum (Represa, et al., 1990). This is in clear contrast to HAT-14, which is detected at high levels in the Purkinje cells of the cerebellum by both *in situ* hybridization (Fig. 18) and immunocytochemistry (Fig. 19). While the difference in localization may reflect some fundamental difference between birds and mammals, it still supports a distinct role for songbird HAT-14 relative to mammalian RC3/neurogranin. It will be interesting to use

the HAT-14 antibodies to probe expression in the rat; e.g., is there a rat HAT-14 homolog that *is* expressed in Purkinje cells?

Some evidence suggests that HAT-14 represents a family of related sequences. When zebra finch brain extracts are blotted and probed with the polyclonal ascites H14-i, generated against an internal peptide sequence present in HAT-14 and RC3/neurogranin, multiple bands are detected whose signal can be preabsorbed by incubation with the specific peptide. These may be either the products of related genes, or the products of alternatively spliced transcripts from the same gene. When Southern blots of zebra finch and canary genomic DNA were hybridized with HAT-14 cDNA probes, smears of signal were inevitably observed (data not shown); this is consistent with the existence of multiple genes sharing HAT-14 sequence, but could also be due some technical problem. On Northern blots of poly(A)$^+$ RNA, a second transcript of ~2000 bp is sometimes observed in addition to the dominant 1000 bp band. The possibility that this is an alternatively spliced HAT-14 transcript or the product of a different gene is as yet untested. Efforts are ongoing in the lab to clone the HAT-14 genomic sequence, which should allow resolution of many of these questions.

A domain common to HAT-14, RC3/neurogranin, and GAP43 is represented in Figure 13. GAP-43 is identical to the protein neuromodulin, the initial purification of which was based upon its ability to bind calmodulin in the absence of Ca^{++} (Cimler, et al., 1985). Subsequent characterization of GAP43/neuromodulin localized the calmodulin binding domain (Alexander, et al., 1988) and a protein kinase C phosphorylation site (Apel, et al., 1990) within the region

now known to be conserved among HAT-14, RC3/neurogranin, and GAP43/neuromodulin. Neurogranin was identified in a subsequent search for protein kinase C substrates in the brain (Baudier, et al., 1989), and has also been demonstrated to bind calmodulin in the absence of Ca^{++} (Deloulme, et al., 1991). Although calmodulin binding and protein kinase C phosphorylation have yet to be demonstrated for HAT-14, the strong conservation of this domain suggests a shared functional or regulatory mechanism among HAT-14, neurogranin, and GAP43. Phosphorylation of GAP43 decreases its affinity for calmodulin (Alexander, et al., 1987a), leading to speculation that GAP-43 may serve to regulate local calmodulin availability dependent upon levels of PKC activity. Changes in calmodulin availability could in turn influence local free Ca^{++} concentration, or other calmodulin-dependent signal transduction events (such as activation of Ca^{++}/calmodulin dependent kinases).

It is also useful to consider the relative localization of HAT-14, neurogranin, and GAP43. GAP43 RNA is low in HVC of adult songbirds (unpublished result), while HAT-14 RNA is notably high. The Ca^{++}-buffering protein parvalbumin is also enriched in HVC relative to surrounding tissue by immunocytochemical localization (Braun, et al., 1991). This is thought perhaps to reflect the high levels of activity characteristic of this nucleus. HAT-14 is also enriched in cerebellar Purkinje cells, likewise notable for their high levels of activity. All three proteins are found in neurons, although GAP43 is localized particularly to axons (Goslin, et al., 1990), while HAT-14 and neurogranin seem predominantly localized to cell bodies

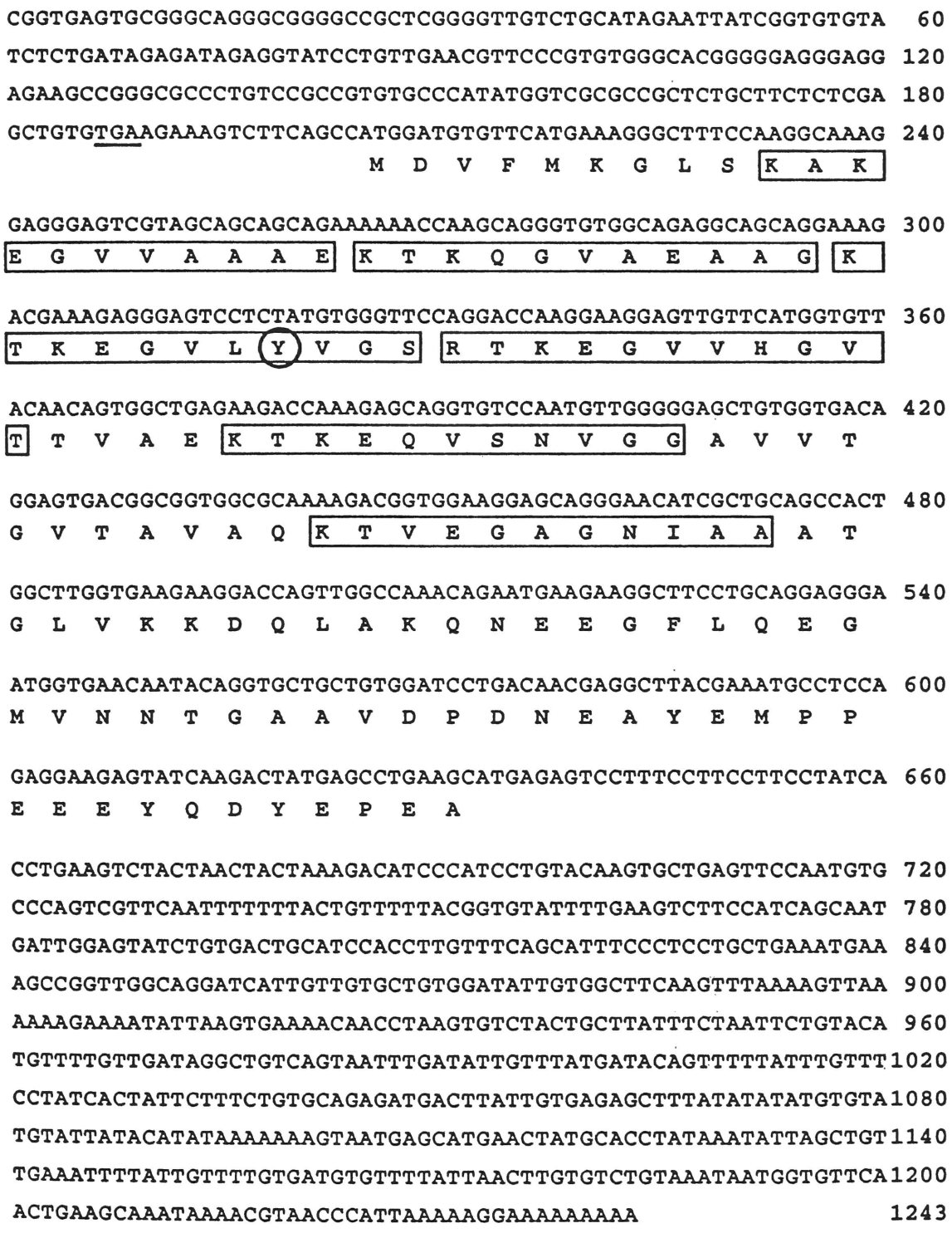
and dendrites (Figure 19), (Represa, et al., 1990). Hence, while sequence comparison suggests conservation of some molecular function among HAT-14, RC3/neurogranin, and GAP43, their patterns of regulation argue for very different functions at the cellular level.

Chapter 6. HAT-3 encodes a novel brain-specific protein conserved in the mammalian brain and differentially regulated in the avian song control circuit

HAT-3 encodes a novel protein with a highly repetitive structure

HAT-3 RNA is ≥4-fold enriched in the HAT relative to non-forebrain, and is present at an abundance of $2x10^{-4}$ (Table 1). The cDNA insert originally cloned and sequenced included 1048 bp of a transcript estimated at 1500 bp. When the sequence of the insert was determined, a likely open reading frame was identified, but it extended unbroken to the extreme 5' end of the sequence. The polymerase chain reaction (PCR) was used to amplify 5' sequences from the HAT cDNA library, relying on the existence of multiple cloned HAT-3 inserts which might randomly include more of the desired sequence. Synthetic DNA primers were designed with specificity for a sequence at the 5' end of the HAT-3 insert and for sequences adjacent to the cloning site of the library vector lambda gt10. These primers were used with purified library DNA to amplify a DNA fragment which was then directly sequenced. From this fragment, an additional 195 bp of 5' HAT-3 sequence were identified. This expanded nucleotide sequence is shown in Figure 21, along with the predicted protein. A stop codon is indicated which

FIGURE 21. Nucleotide sequence and predicted amino acid product of HAT-3 cDNA, with boxed degenerate repeat. The full HAT-3 cDNA sequence, including 195 bp generated by PCR-based sequencing of the HAT cDNA library. Degenerate repeats within the sequence are indicated by boxes. A 5' stop codon limits the open reading frame to that presented. A tyrosine residue which is a potential target for phosphorylation is circled.


CGGTGAGTGCGGGCAGGGCGGGGCCGCTCGGGGTTGTCTGCATAGAATTATCGGTGTGTA 60
TCTCTGATAGAGATAGAGGTATCCTGTTGAACGTTCCCGTGTGGGCACGGGGGAGGGAGG 120
AGAAGCCGGGCGCCCTGTCCGCCGTGTGCCCATATGGTCGCGCCGCTCTGCTTCTCTCGA 180
GCTGTGTGAAGAAAGTCTTCAGCCATGGATGTGTTCATGAAAGGGCTTTCCAAGGCAAAG 240
M D V F M K G L S K A K
GAGGGAGTCGTAGCAGCAGCAGAAAAAACCAAGCAGGGTGTGGCAGAGGCAGCAGGAAAG 300
E G V V A A A E K T K Q G V A E A A G K
ACGAAAGAGGGAGTCCTCTATGTGGGTTCCAGGACCAAGGAAGGAGTTGTTCATGGTGTT 360
T K E G V L Y V G S R T K E G V V H G V
ACAACAGTGGCTGAGAAGACCAAAGAGCAGGTGTCCAATGTTGGGGGAGCTGTGGTGACA 420
T T V A E K T K E Q V S N V G G A V V T
GGAGTGACGGCGGTGGCGCAAAAGACGGTGGAAGGAGCAGGGAACATCGCTGCAGCCACT 480
G V T A V A Q K T V E G A G N I A A A T
GGCTTGGTGAAGAAGGACCAGTTGGCCAAACAGAATGAAGAAGGCTTCCTGCAGGAGGGA 540
G L V K K D Q L A K Q N E E G F L Q E G
ATGGTGAACAATACAGGTGCTGCTGTGGATCCTGACAACGAGGCTTACGAAATGCCTCCA 600
M V N N T G A A V D P D N E A Y E M P P
GAGGAAGAGTATCAAGACTATGAGCCTGAAGCATGAGAGTCCTTTCCTTCCTTCCTATCA 660
E E E Y Q D Y E P E A
CCTGAAGTCTACTAACTACTAAAGACATCCCATCCTGTACAAGTGCTGAGTTCCAATGTG 720
CCCAGTCGTTCAATTTTTTTACTGTTTTTACGGTGTATTTTGAAGTCTTCCATCAGCAAT 780
GATTGGAGTATCTGTGACTGCATCCACCTTGTTTCAGCATTTCCCTCCTGCTGAAATGAA 840
AGCCGGTTGGCAGGATCATTGTTGTGCTGTGGATATTGTGGCTTCAAGTTTAAAAGTTAA 900
AAAAGAAAATATTAAGTGAAAACAACCTAAGTGTCTACTGCTTATTTCTAATTCTGTACA 960
TGTTTTGTTGATAGGCTGTCAGTAATTTGATATTGTTTATGATACAGTTTTTATTTGTTT 1020
CCTATCACTATTCTTTCTGTGCAGAGATGACTTATTGTGAGAGCTTTATATATATGTGTA 1080
TGTATTATACATATAAAAAAAGTAATGAGCATGAACTATGCACCTATAAATATTAGCTGT 1140
TGAAATTTTATTGTTTTGTGATGTGTTTTATTAACTTGTGTCTGTAAATAATGGTGTTCA 1200
ACTGAAGCAAATAAAACGTAACCCATTAAAAAGGAAAAAAAAA 1243

constrains the translation product to that shown. Sequences preceding methionine #1 favor it as the start site for translation when compared to sequences near methionine #5, according to the consensus established by Kozak (Kozak, 1986), although this should not be regarded as conclusive.

No HAT-3 homolog has been found in any database, although sequence comparisons with FASTA sequence analysis software detect some distant resemblance to a plant protein, LEA76, and to a surface antigen described in the protozoan Plasmodium falciparum. Each of these foreign sequences is notable for the presence of variable tandem repeats(Cowman, et al., 1985),(Harada, et al., 1989). Upon closer inspection, HAT-3 was found also to contain an 11 amino acid domain which is degenerately repeated (i.e., with conservative amino acid substitutions within the repeats) six times in the peptide sequence (boxed residues in Figure 12).

A dot matrix alignment of HAT-3 with the plant protein LEA76 is shown in Figure 22. Diagonal lines represent clusters of conserved residues, and groups of parallel diagonal lines represent the repeats. While the repeat is confined to the first 100 amino acids of HAT-3, similarity to LEA76 continues to the end of the molecule. This may indicate a level of structural conservation between the two proteins which does not arise exclusively from the common repeated element.

There is a very regular variation in hydrophobicity over the length of the HAT-3 peptide sequence, as shown graphically in Figure 23. This periodic structure is determined primarily by the repeats: the beginning of each repeat is composed of hydrophilic

FIGURE 22. Dot matrix alignment of HAT-3 with the plant protein LEA76. A sliding window compares every 9 amino stretch of the HAT-3 sequence (aligned along the vertical axis) across the entire LEA76 sequence (horizontal axis), and a dot is placed wherever identities are found. Diagonal lines indicate extended regions of similarity, and parallel lines indicate these regions are repeated at multiple sites.

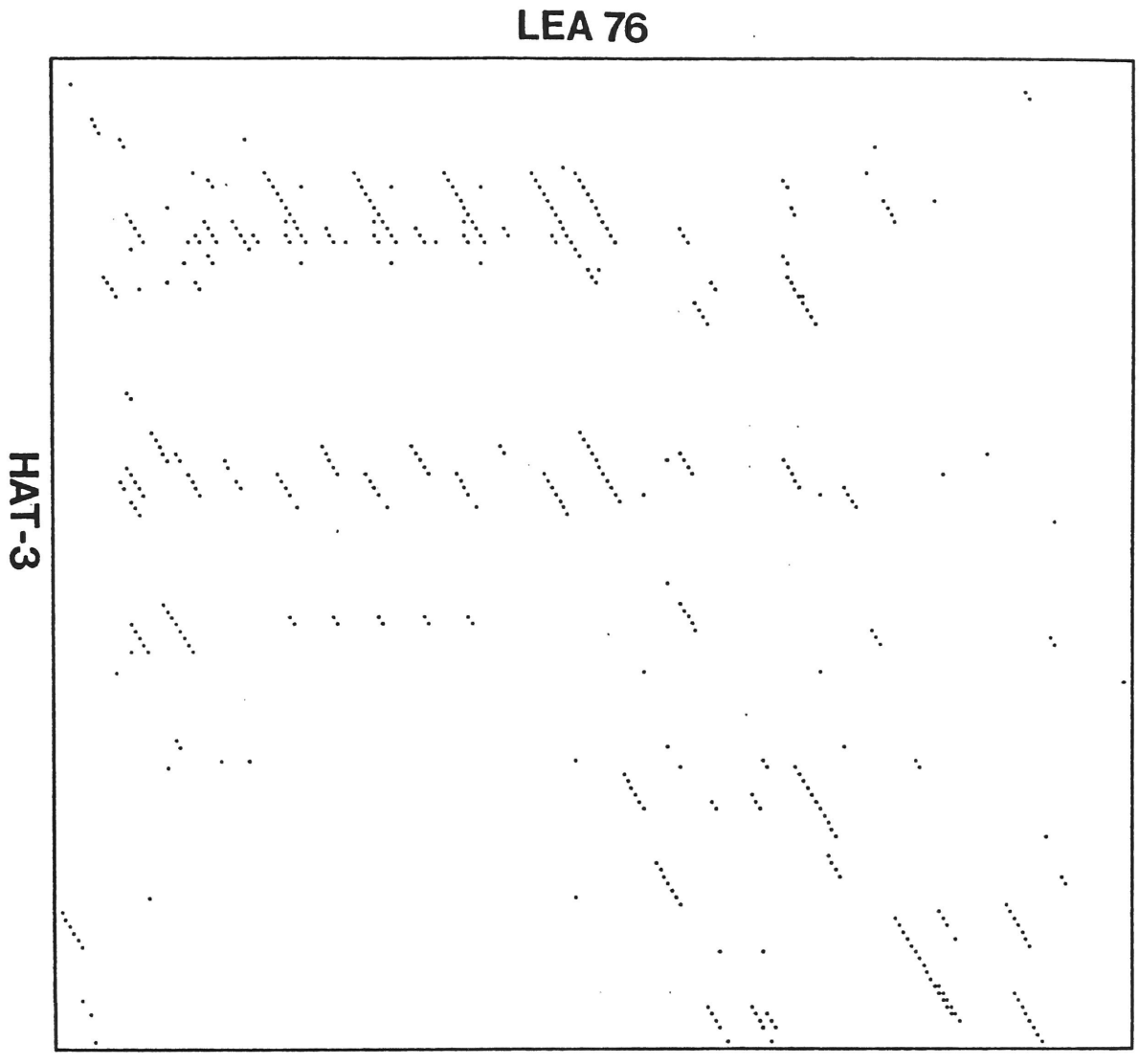
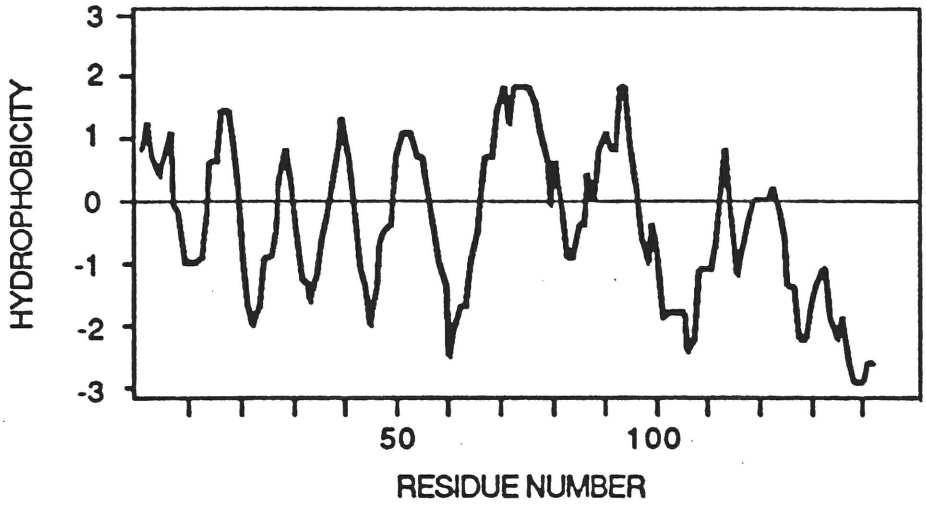

LEA 76
HAT-3

FIGURE 23. Hydrophobicity plot of the HAT-3 predicted peptide, using the algorithm of Kyte and Doolittle, 1982. Periodic variations in hydrophobicity in the first 100 residues correspond to the placement of the 11 amino acid repeat.

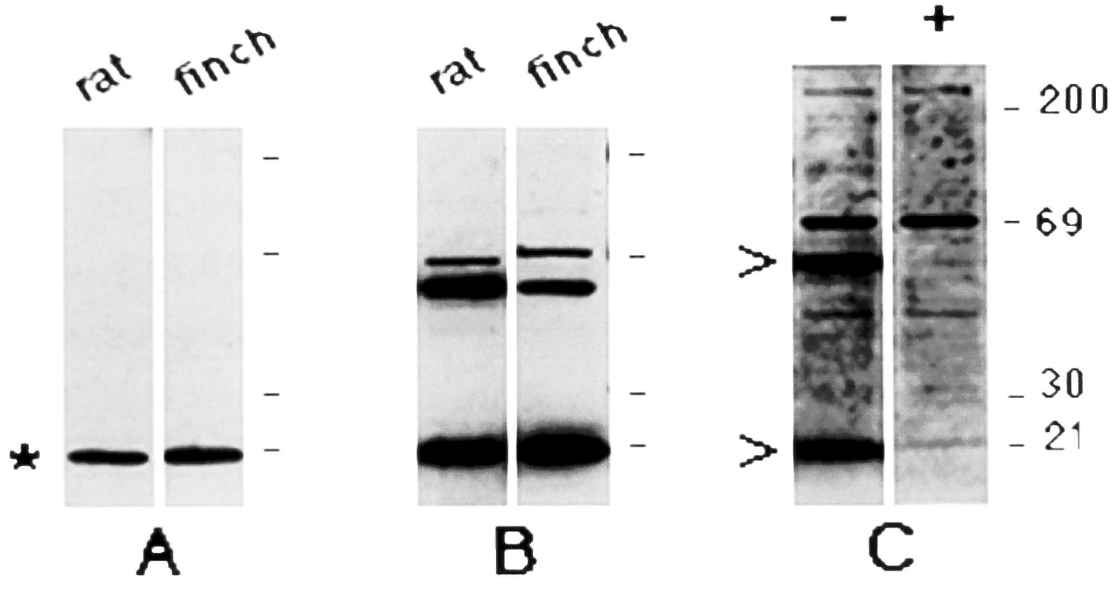

HYDROPHOBICITY
3
2
1
0
-1
-2
-3
50
100
RESIDUE NUMBER

residues, with a transition to more hydrophobic residues toward the end.

Antipeptide antibodies detect species of 17 and 65 kD in extracts of both zebra finch and rat brain

To confirm that the protein encoded by HAT-3 is actually produced, antisera were raised against the peptide (C E M P P E E E Y Q D Y E P E A), which represents the 15 carboxy terminal residues of HAT-3 plus an amino-terminal cysteine to allow coupling to a carrier protein. After conjugation with the carrier KLH, the peptide was used to immunize mice. Sera were screened against HAT-3 protein expressed in E. coli with the pMAL vector system (New England Biolabs), and polyclonal ascites were generated in positively reactive mice. Antibodies were used to probe crudely fractionated extracts of zebra finch and rat brain on Western blots followed by chemiluminescent detection (ECL, Amersham). In the blots shown in Figure 24, all lanes contain the crude synaptosomal fraction. Panels A and B represent antibodies raised against the HAT-3 peptide in two different mice. In Panel A, a single protein of similar size is detected in both rat and finch extracts. Its size is not well-resolved here, but under different separating conditions the band seems to migrate at about 17 kD (data not shown). In Panel B, multiple protein species are detected, including conserved bands at 17 and 60 kD, and a third band which migrates at about 67 kD in rat and 70 kD in finch. The 67 and

FIGURE 24. Western analysis of HAT-3 immunoreactivity in synaptosomal fractions of zebra finch and rat brain. Each panel shows lanes from a single gel after blotting, antibody incubation and detection via chemiluminescence. Common size markers are indicated by the dashes (sizes in kD given by panel C). **A)** Antiserum from mouse "L" reacts with a single band (*), detected in synaptosomal extracts from both rat and finch forebrain. **B)** Antiserum from mouse "O" also reacts with larger molecular weight bands in both rat and finch (the shift in size of the largest band in rat vs. finch is reproducible). **C)** Competitive adsorption experiment (finch): normal antiserum "O" (left lane, "-") reacts with multiple bands as in panel B, but after pre-incubation with the immunizing peptide (right lane, "+") it no longer reacts with the primary band or the dominant higher molecular weight band (indicated by ">").


rat
finch
rat
finch
-
+
200
69
30
21
A
B
C

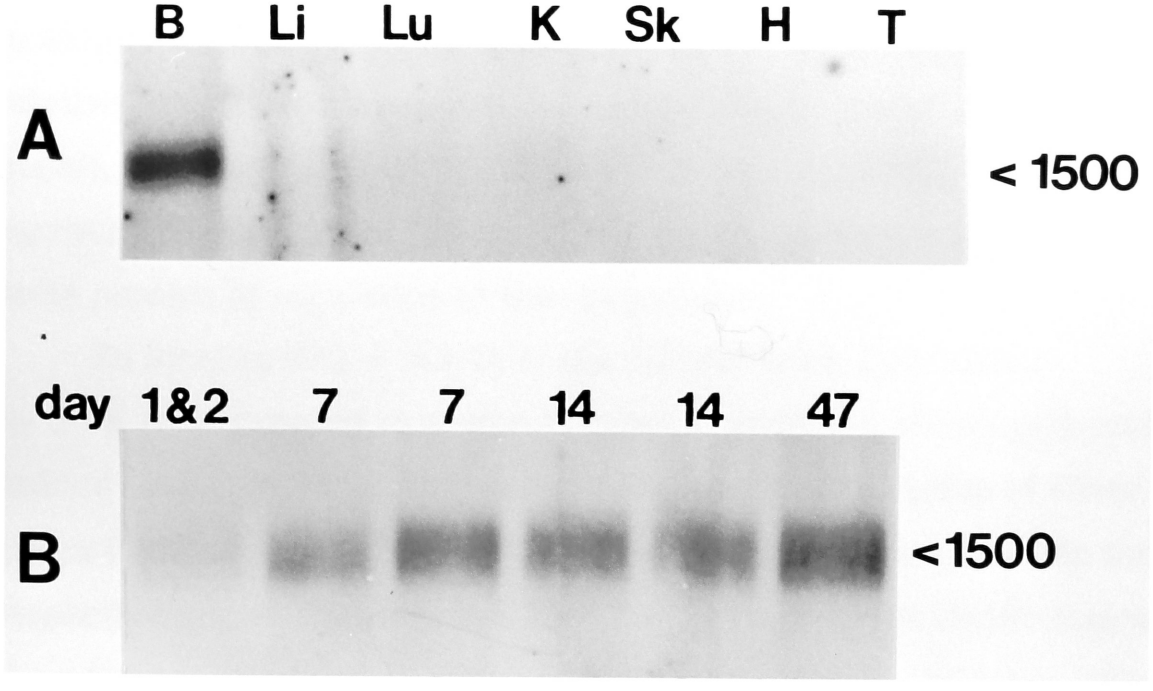
70 kD bands are not detected in the cytosolic fractions, which otherwise display a similar qualitative and quantitative distribution of HAT-3 immunoreactivity (data not shown). Signal for the 17 and 60 kD bands can be preabsorbed by incubation with the HAT-3 peptide, while signal for the larger band is unchanged. This suggests that the higher molecular weight species is non-specifically cross-reactive, while the 17 and 60 kD bands are specific.

Northern blot analysis suggests that HAT-3 is brain-specific and developmentally regulated in young zebra finches

The HAT-3 cDNA was used to probe Northern blots of poly(A)$^+$ RNA representing a variety of canary tissues including forebrain, liver, lung, kidney, skeletal muscle, heart, and testes (Fig. 25, Panel A). The HAT-3 transcript was detected only in brain. Identical blots probed for actin demonstrated the presence of the ubiquitous β-actin transcript in all tissues, and additional actin isoforms in heart, skeletal muscle, and kidney (see Fig. 4, Panel B).

Northern analysis was used to determine the developmental onset of HAT-3 expression in the zebra finch forebrain. Poly (A)$^+$ RNA was isolated from the forebrains of zebra finches of known age, blotted, and probed with the HAT-3 cDNA (Fig 25, Panel B). There is little transcript detected at 1-2 days post-hatch, with a large increase in signal by 7 days. A modest increase in signal occurs between 7 and 47 days. Overall signal in the forebrain at 47 days resembles that seen in the adult (data not shown). It thus appears that most broad regulation of the HAT-3 transcript is occuring during the first

FIGURE 25. Northern analysis of HAT-3 mRNA in zebra finch tissues and developing zebra finch forebrain. **Panel A:** each lane represents 1 microgram polyA(+) RNA isolated from canary tissues (l. to r., forebrain, liver, lung, kidney, skeletal muscle, heart and testes). A single band of 1500 nucleotides is detected only in brain. Identical blots probed with actin show hybridization in all lanes (see Fig. 16). **Panel B:** Developmental profile of HAT-3 RNA expression. Each lane represents 1 microgram poly(A)+ RNA isolated from the forebrain of a single different zebra finch of the indicated age, except for the 1-2 day sample, in which a 1-day and a 2-day sample were pooled.


B
Li
Lu
K
Sk
H
T
A
< 1500
day
1&2
7
7
14
14
47
B
<1500

week post-hatch, a period of immense brain growth and synaptogenesis (Herrmann and Bischof, 1986).

HAT-3 mRNA is regulated in the avian song circuit

HAT-3 mRNA was localized in the songbird forebrain using *in situ* hybridization. Figure 26 shows a section from a male canary in Spring. HAT-3 signal is high generally in the forebrain, particularly in neo- and hyperstriatum, and relatively low in the midbrain, cerebellum, and optic tectum. This section is somewhat overexposed to emphasize the low signal in song nuclei LMAN, RA, and HVC relative to surrounding tissue. As described previously (Fig. 5, lower panel), control probes do not distinguish the song nuclei from surrounding tissue, and no other HAT mRNA shares precisely the same pattern of regulation in the song circuit.

To localize HAT-3 mRNA at the cellular level, hybridized sections were exposed to autoradiographic emulsion, developed, and stained with cresyl violet. Figure 27 shows the distribution of silver grains within song nuclei LMAN and RA and immediately outside the respective nuclei. There is less labelling per cell inside LMAN than in the adjacent neostriatum. Signal is observed over several large cells with neuronal profiles, suggesting that the RNA is expressed in neurons, although expression in glia is possible as well. RA likewise shows less labelling per cell than in adjacent archistriatum. Examples of relatively unlabelled neurons are evident in both song nuclei, suggesting that HAT-3 expression may be restricted to particular subsets of cells.

FIGURE 26. Distribution of HAT-3 mRNA in sagittal sections of adult male canary brain.Note sharply reduced signal in the 3 song nuclei HVC, RA and lateral MAN compared to surrounding striatal regions. (The 2 small holes at the posterior pole of the forebrain are tears in the tissue).

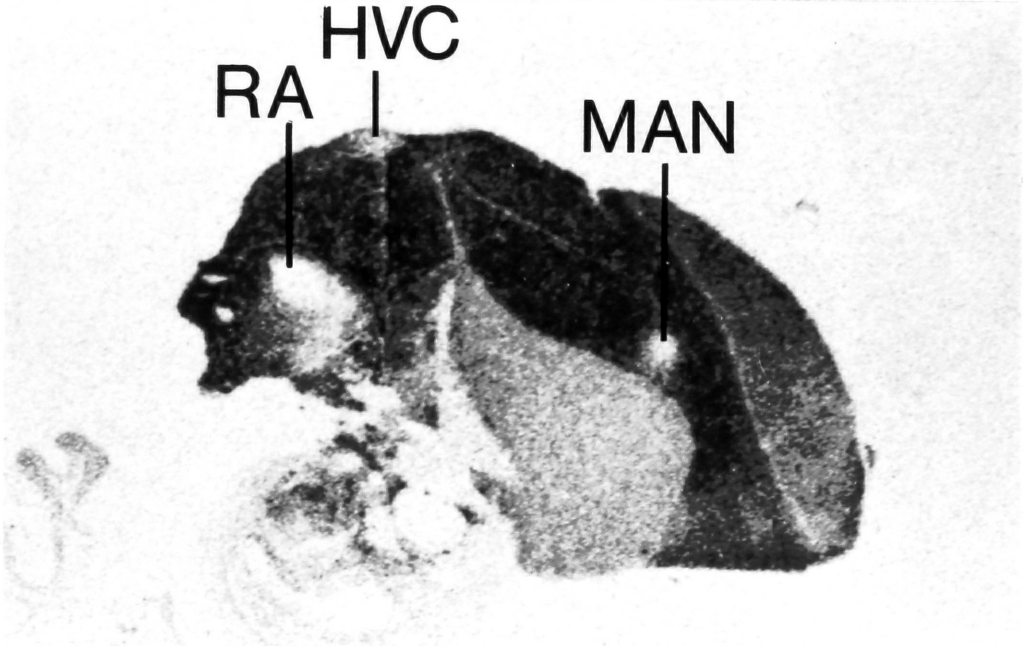
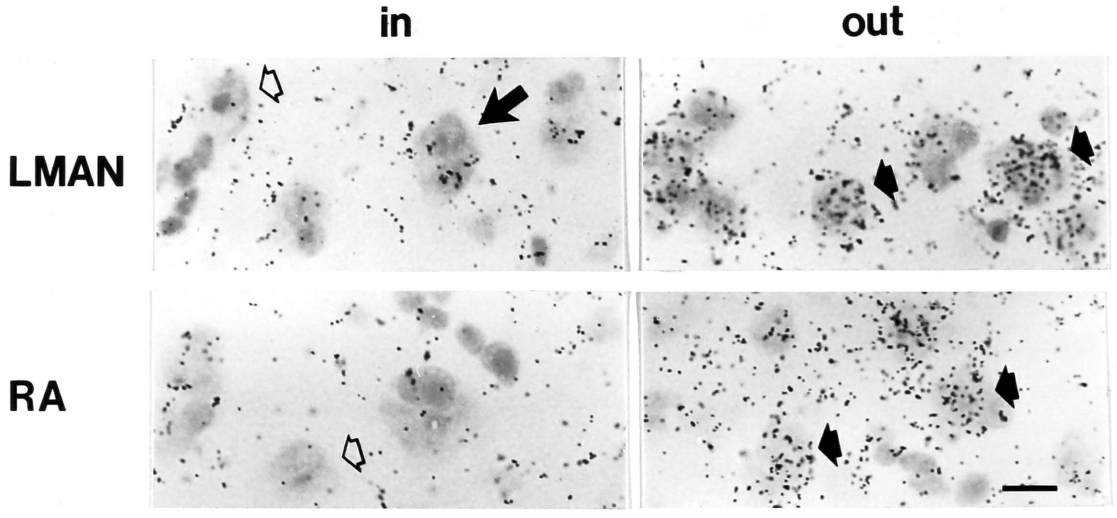

HVC
RA
MAN

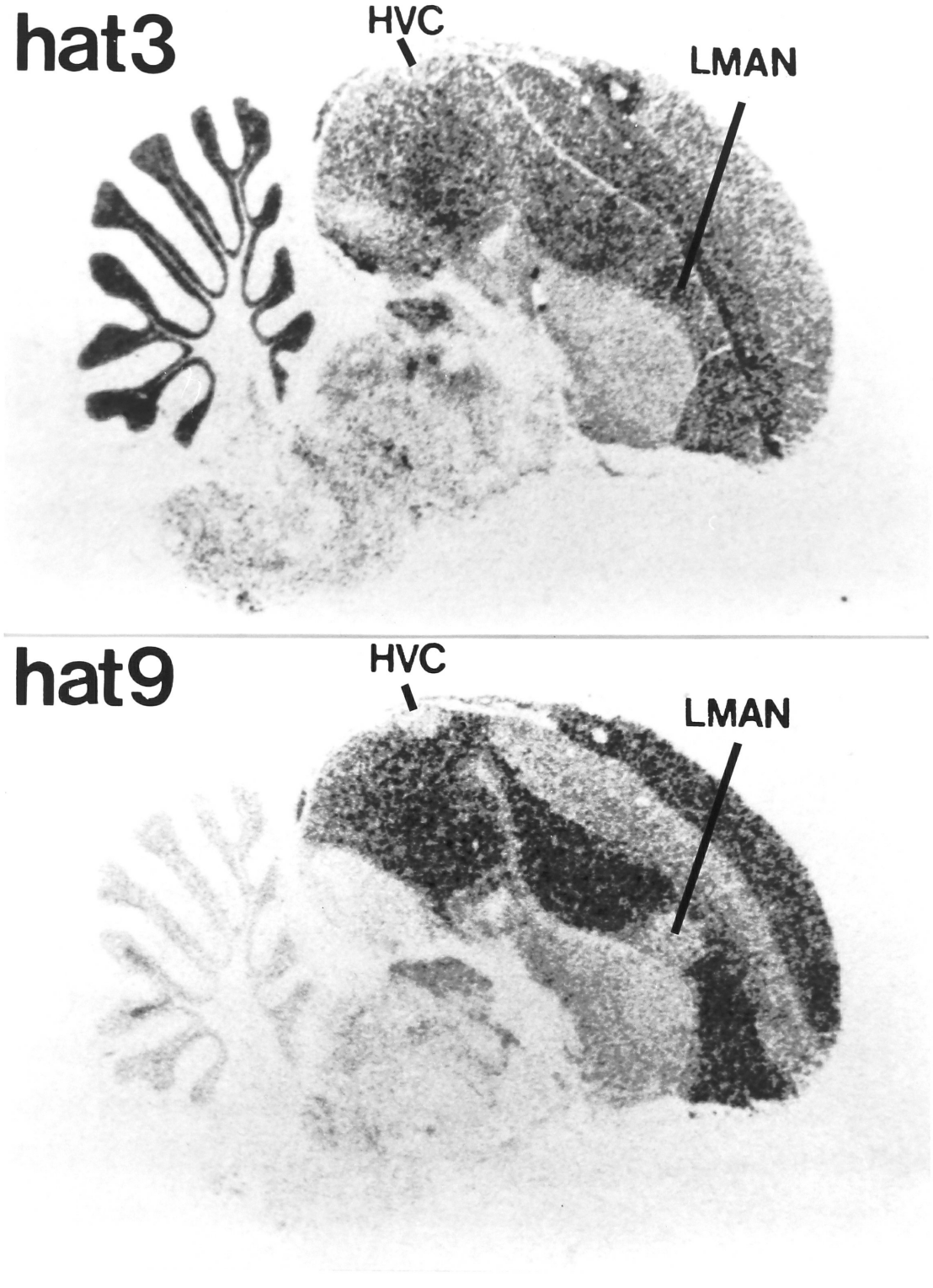
FIGURE 27. Cellular expression of HAT-3 mRNA in song nuclei lateral MAN and RA (bright field emulsion autoradiographs). Sections hybridized with 35S-labelled HAT-3 riboprobes were exposed to autoradiographic emulsion, developed and counterstained with cresyl violet. Fields inside and immediately outside lateral MAN and RA (respectively) are compared (see text for discussion). Size bar: 10 microns.


in
out
LMAN
RA

Since low levels of HAT-3 RNA expression were observed in the song nuclei HVC, RA, and LMAN of adult male canaries in Spring (Fig. 26) and adult male zebra finches (data not shown), each of which represents a period of relatively low song plasticity and high song production, it was speculated that levels of HAT-3 RNA might be higher at times corresponding to greater song plasticity. Sections from male canaries sacrificed in Spring (March-May) and in Fall (October) were hybridized with the HAT-3 probe and exposed to X-ray film. There were no obvious differences between the patterns of HAT-3 expression observed at these points, although a more refined time course throughout the year would be required to conclude that HAT-3 expression does not change.

However, a comparison of zebra finches of different ages revealed a change in HAT-3 expression which correlates with the critical period for song development in this species. While a "hole" in HAT-3 expression in LMAN is clearly evident by 60 days (data not shown), a peak in HAT-3 expression relative to surrounding neostriatum is observed in LMAN at 15 and 25 days (3 birds at each age). Figure 28 shows a 25 day-old zebra finch hybridized for HAT-3 and another HAT clone, HAT-9 (see Table 1). There is a "hole" for HAT-9 expression in LMAN at all ages observed (data not shown), and for this reason it is a useful control and marker for the song circuit. Another notable developmental change occurs in the cerebellum: while signal in cerebellar granule cells is as high or higher than forebrain signal at 25 days, it is much lower in adult canaries and zebra finches (compare Figs. 26 and 28).

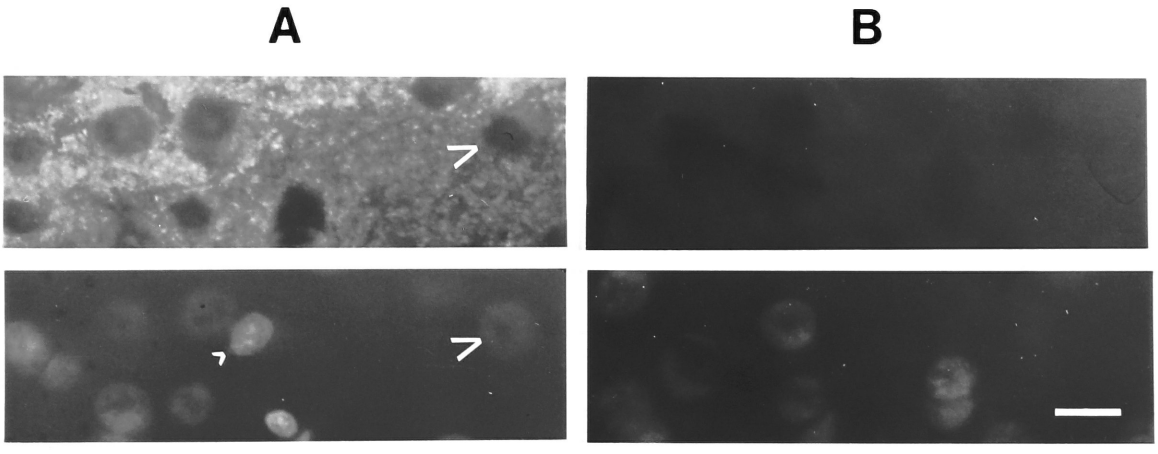
FIGURE 28. Distribution of HAT-3 mRNA (top) compared with HAT-9 (bottom), as detected by film autoradiography in adjacent sagittal sections of 25-day-old zebra finch. See text for discussion.


hat3
HVC
LMAN
hat9
HVC
LMAN

Immunocytochemistry localizes HAT-3 protein to neuropil

HAT-3 protein was localized in perfusion-fixed, PEG embedded tissue from the zebra finch brain. The tissue was probed with polyclonal ascites H3-1 (Figure 24, Panel A) followed by a fluorescein-conjugated goat anti-mouse secondary antibody and visualized by fluorescence microscopy. Figure 29 shows comparable fields within the song nucleus Area X (for which no "hole" in HAT-3 RNA is observed) stained with either H3-1 or secondary antibody only (upper panels, A and B respectively). For HAT-3, dense punctate staining is evident, interspersed with spots which are devoid of staining. Control signal is limited to a barely visible haze, although both fields stain comparably with a counterstain for cell nuclei (lower panel). As demonstrated by the counterstain, most of the spots which are devoid of staining correspond to the positions of cell nuclei. This suggests that the antigen is excluded either from the nucleus or from the entire soma of these cells. Several of these nuclei are relatively large and of low density, suggesting that they belong to neurons (large arrows). Conversely, most nuclei correspond to areas of low antibody staining, although an exception is highlighted in the lower panel of A. A bright, densely staining nucleus is observed (small arrow) which shows no corresponding patch of low somatic staining in the upper panel. This may represent a glial cell which, unlike the neuron above, contains HAT-3 antigen within its soma. Alternatively, the soma may just appear to be stained because of the presence of stained material in another plane of focus.

FIGURE 29. Immunocytochemical localization of HAT-3 in Area X. Panels A and B represent identical fields within Area X of adjacent sections, stained respectively with HAT-3 ascites and with fluorescent secondary antibody only. The lower panels represent nuclear counterstaining detected in another fluorescence channel (see text for discussion). Size bar: 10 microns.


A
B

To better understand the origins of this patchy pattern of staining, cells from the brains of 3-day-old zebra finches were cultured for 3 days, fixed on coverslips and stained with either H3-O or non-immune mouse serum (Figure 30, a and b) A low level of background is observed in the control cultures, while bright staining of cell processes is evident in the cultures stained with H3-O. When cells from a 1-day-old bird were cultured at lower density for 6 days, an additional staining characteristic was noted. A distinctly punctate component was observed in neuronal cell bodies, frequently polarized to the base of a neurite (Panel c). When compared to the staining pattern observed in tissue at the same magnification (Panel d), the bright spots observed in these two samples were comparable in size and brightness.

Figure 31 shows another field from the same stained culture as Panel c, above. A neuron on the lower left extends a process which grows brighter toward its distal tip. The staining abruptly declines near the cell on the upper right, but a faintly staining process is still visible which completes the connection between the two cells. It is not clear whether the less-stained process is a continuation of the bright process or in fact represents a different process emanating from the cell on the upper right. In addition, this second cell extends a more brightly staining process in the opposite direction, ending in what appears to be a growth cone. Punctate staining is observed at the bases of all three neurites, particularly the two which are longer are more brightly stained. This suggests an association of HAT-3 with process outgrowth.

FIGURE 30. Immunofluorescent localization of HAT-3 peptide in primary cultures of zebra finch telencephalon. **Panel A:** stained with antibody to HAT-3 followed by fluorescein-conjugated secondary antibody; **B:** similar field stained with with secondary antibody only; **C:** lower density culture stained for HAT-3; **D:** for comparison, a section of zebra finch brain (in Area X) is stained for HAT-3. All 4 fields are at same magnification (size bar: 10 microns). Note staining of processes in A, and similar punctate staining in cells and tissue in C and D.

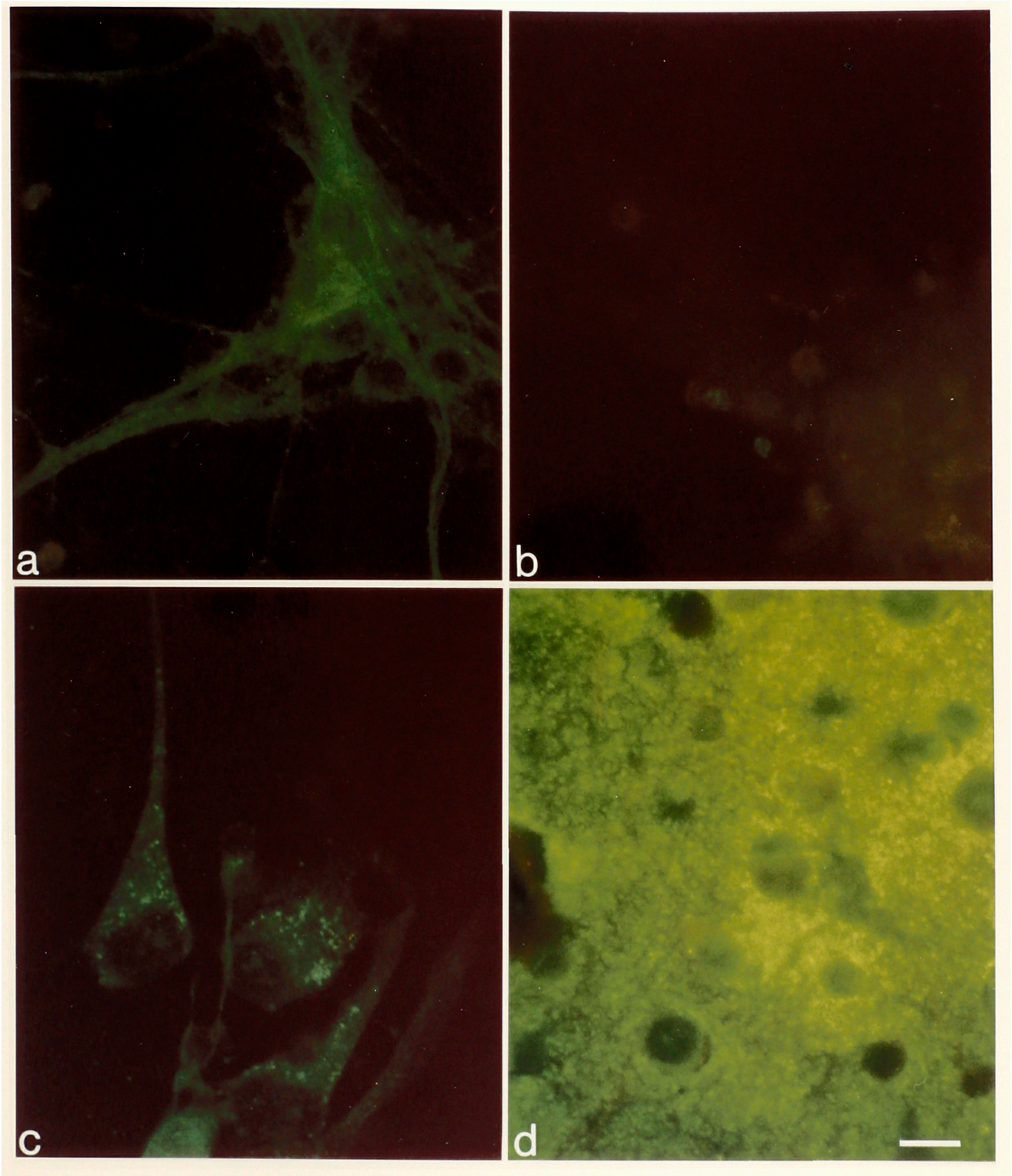
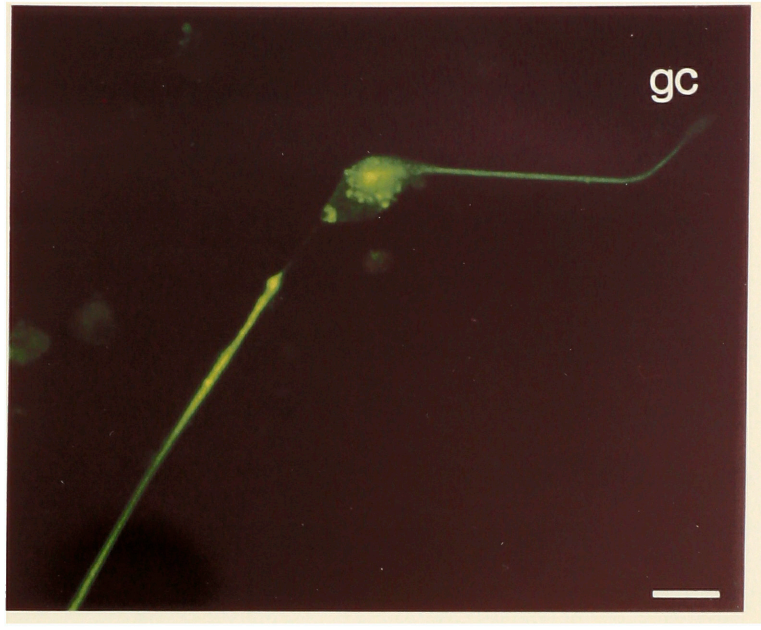
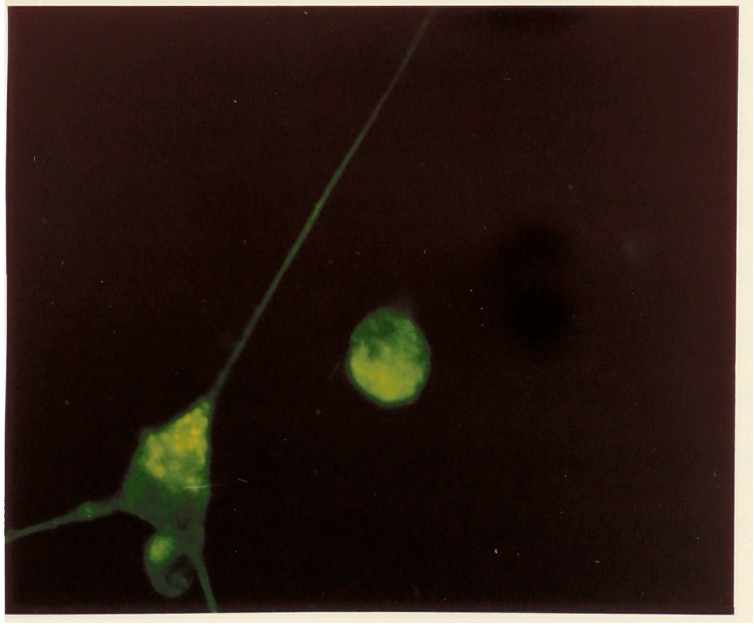

a
b
c
d

FIGURE 31. Immunofluorescent localization of HAT-3 in low density primary culture. A single cell in the lower left corner extends a long process which continues into the field shown in the upper right panel; the placement of the 2 fields in the photograph represents their approximate relationship and distance in the culture. "GC" indicates a growth cone. See text for further discussion. Size bar: 10 microns.


gc



Discussion

HAT-3 is a novel, brain-specific RNA, the levels of which increase generally in the forebrain during development. HAT-3 RNA distribution was examined in the brains of two songbird species at times characterized by high song production and low song plasticity: adult male canaries in Spring, and adult male zebra finches. In both species, HAT-3 RNA is distributed rather widely in the forebrain, particularly in neo- and hyperstriatum, the avian equivalent of the mammalian cortex. However, levels of HAT-3 RNA are very low in the song control nuclei HVC, RA, and LMAN, relative to surrounding tissue. These three nuclei are known to concentrate gonadal steroid hormones (Arnold, et al., 1976), (Balthazart, et al., 1992), which regulate song development and production in both species (Arnold, 1975), (Alvarez-Buylla, et al., 1987).

The initial correlation of very specific "holes" in HAT-3 RNA expression with periods of low song plasticity led to the hypothesis that HAT-3 expression in the song nuclei might vary with the plasticity of the animal. To test this, adult canaries were examined at two times during the year: Spring (March-May) and Fall (October), the latter characterized by high plasticity and low song stability. The pattern of HAT-3 distribution is not obviously different at these two times, although transient changes or changes occurring at other times during the year cannot be excluded.

However, a change in HAT-3 RNA expression does occur in the song circuit of young zebra finches. While HAT-3 RNA is clearly a "hole" in LMAN by 60 days, a peak in HAT-3 RNA is observed in this

nucleus at 15 and 25 days. This result is very compelling because of numerous experiments linking LMAN specifically with song development as opposed to maintenance of mature song. Lesions of LMAN disrupt singing behavior during the critical period for song learning, but such lesions have no effect on song once the pattern of notes has stabilized at about 55-60 days (Bottjer, et al., 1984). When the critical period for song learning is extended by maintaining young birds in visual isolation, susceptibility of the song to LMAN lesion is likewise extended (Morrison and Nottebohm, 1990). LMAN regresses over the course of song development, with a net loss of half of the its original neurons (Bottjer, et al., 1985). These observations suggest that LMAN is most important during the early stages of song development, the time when HAT-3 expression in this nucleus is also high. Further experiments might determine whether the decrease in HAT-3 RNA expression is due to the selective loss of HAT-3 expressing cells, or to other factors.

A recent review article includes the unpublished observation that LMAN makes its projection to RA at about 15-20 days post-hatch (Bottjer and Johnson, 1992), corresponding with a period of high HAT-3 RNA expression in LMAN at 15 and 25 days. This and other observations support the hypothesis that HAT-3 has a role in process outgrowth or synapse formation. The HAT-3 protein is present at high levels in crude synaptosomal fractions of zebra finch and rat brain extracts, in contrast to HAT-14 (previous Chapter) which is clearly enriched in cytosol relative to synaptosome. Immunocytochemistry in sections of zebra finch brain localize the protein primarily to punctate spots in the neuropil, with relatively

little staining evident within cell bodies. This pattern resembles quite strikingly the pattern published for antibodies to synaptotagmin (Wendland, et al., 1991), a synaptic vesicle protein characterized in rat and marine ray. Double-labelling with antibodies to HAT-3 and synaptotagmin could clearly establish the coincidence of these proteins and provide support for the synaptic localization of HAT-3. Finally, experiments in primary neuronal cultures suggest an association of HAT-3 with growing neurites. It would be interesting to see whether addition of HAT-3 antibodies to the culture medium would affect the morphology and behavior of these cells. At the organismal level, a test of HAT-3 function might be devised by injecting antisense oligonucleotides to "knock out" HAT-3 expression in LMAN of young zebra finches, then assaying for effects process outgrowth and song development.

Chapter 7. Conclusions and Implications

In the preceding chapters, I presented the detailed characterization of 4 mRNAs (and their predicted peptides) that were isolated by virtue of their enrichment in the neo- and hyperstriatum surrounding and including song nucleus HVC of adult male canaries. To summarize the salient properties of each --

HAT-2: this RNA is brain-specific, and encodes a novel signal transduction protein apparently sensitive to diacylglyerol-mediated signals and capable of influencing the GTPase activity of other proteins, such as *rac*. A related protein has been detected in the human neocortex; strong evolutionary conservation of both sequence and anatomical pattern of expression suggests this molecule has a fundamental role in the function of neurons in the telencephalon. In the avian song circuit, the RNA is notably less abundant in the androgen concentrating song nuclei of the forebrain relative to the immediately surrounding brain.

HAT-5: this RNA is enriched in brain but expressed in other tissues as well; it encodes the apparent canary homologue of MEK1 (MAP-kinase activator), a key protein kinase in the pathway transducing growth factor stimulation into intracellular events such as gene activation. In the songbird the RNA is expressed widely in the brain, somewhat enriched in the forebrain and somewhat less

abundant in HVC. The RNA appears to be present both in mature neurons and in the neurogenic ventricular zone.

HAT-14: this RNA is brain-specific, and among the HATclones (Table 1) is the most HAT-enriched, despite its high level of expression in a restricted set of cells in the cerebellum. The RNA encodes a novel protein that is related (but not necessarily equivalent) to a recently described mammalian forebrain-enriched protein (RC3/neurogranin), and more distantly related to GAP43. Common to all of these proteins is a distinctive domain which appears to integrate protein kinase C signalling with calmodulin availability. This is the only one of the HATclones that is actually enriched in HVC compared to surrounding tissue, and is also enriched in L-MAN and less abundant in Area X. The protein appears to be localized to dendrites, a characteristic especially evident in cerebellar Purkinje cells.

HAT-3: this brain-specific RNA encodes a novel protein with a distinctive structure and pattern of regulation, highly suggestive of a function in process outgrowth and/or synaptic structure. Observations which support this hypothesis include:

> the presence of a repeated element in the protein which, based on physiochemical grounds and similarities to other proteins in plants and parasites, might facilitate interactions with membranes or other proteins.

> repression of the RNA in the androgen-concentrating song nuclei of adult birds, and transiently higher levels of

expression correlated with development of the song circuit of zebra finches.

> localization of the protein to neuropil in sections of tissue, and to growing neurites in neuronal cultures.

Implications for Songbird Neurobiology

The song circuit has evolved specifically in birds which learn their vocalizations (oscine passerines) and is absent from birds of the same taxonomical order which do not learn their vocalizations (non-oscine passerines). This suggests a relationship between 1) the specific patterns of gene regulation which are characteristic of the song circuit, and 2) the ability to learn and modify song.

In each of the 4 cases examined here (and also with HAT-9), the song nuclei "stand out" (in greater or lesser degree) from the rest of the forebrain in their pattern of expression of these RNAs. There is no simple or consistent pattern of regulation within the song system, however. Perhaps surprisingly, despite the fact that these clones were selected by their enriched expression in the HAT, we see more examples of *decreased* expression in HVC and the other telencephalic nuclei, at least compared to surrounding forebrain. This raises the tentative notion that the song nuclei are in some way less "forebrain-like" and more similar to lower brain regions.

The song control circuit is usually considered to be an example of a very "plastic" system. Yet we have seen no evidence so far for variations in gene expression in Fall vs. Spring canaries, and evidence

in the finch only for changes at the earliest points of circuit development. In related work in David Clayton's lab, expression of at least 2 molecules associated with growth and plasticity (GAP43 and ZENK, the homologue of an "immediate early" transcription factor) has been found to be unexpectedly low in the song nuclei. Similarly, NMDA receptors appear to be relatively impoverished in the song nuclei (Aamodt, et al., 1992). Thus molecules that are associated with plasticity are paradoxically decreased in this plastic circuit. One possible explanation is that a distinctive set of mechanisms may be responsible for plasticity in the song nuclei, and we have yet to identify any of the molecules responsible. A second possibility is that general mechanisms of plasticity are in fact repressed in the song circuit (or at least the primary song nuclei HVC, RA, LMAN and Area X), perhaps to allow the long-term maintance of learned song patterns, or to permit very specific activation of plastic change at discrete times in development. In the latter case it may be that increased expression of molecules like HAT-3 (or GAP43) would occur in the song nuclei but only during very small windows which have not been identified yet.

Implications for Gene Expression in the Brain

The seven HATclones analyzed here represent a sample of a larger population of genes expressed in the brain, and specifically of those enriched in the neo- and hyperstriatum, avian brain regions considered homologous to mammalian cortex. Consideration of previous work characterizing the general attributes of the population

of RNAs expressed in the canary forebrain (Clayton and Huecas, 1990) suggests that perhaps 40 or 50 RNAs exist that are expressed at similar levels of abundance (.001% - .1%) and with a similar "HAT-enriched" distribution as described for these 7 clones (D. Clayton, unpublished).

It is interesting that at the time this work began, none of these seven sequences was recognizeable based on identity to known sequences (although specific functional domains were apparent in each). In the last 2 years, however, sequences have been published which are closely related to three of them (HAT-2, -5, and -14). The two sequences that remain the most inscrutable (HAT-1 and HAT16) are also the most rare. This suggests that the molecular biology community may be closing in on a complete description of the population of molecules expressed at moderate levels in the brain, but that the much larger population of molecules expressed at very low levels (Milner and Sutcliffe, 1983; Clayton and Huecas, 1990) remains much more poorly characterized. (It is also possible these rarer molecules have been much more poorly conserved in evolution).

Of the 4 sequences characterized in detail here, it is also interesting that 3 of them almost certainly function in signal transduction (HAT-2, -5, and -14), and all of them have features suggesting roles in neural plasticity. It seems likely that signal transduction and neural plasticity represent two especially prevalent functions in the telencephalon in which these RNAs are enriched.

****

Insofar as functional hypotheses drive the identification of novel gene products in the brain, we are limited to discovering molecules that fit our current assumptions about neural function. The tremendous complexity of the brain in terms of cell type, synaptic specificity, and capacity for signal integration suggests that we may have barely scratched the surface in our understanding of the nervous system. Continued application of the powerful techniques of molecular analysis, in rich biological systems like the songbird, promises to yield unexpected discoveries.

Bibliography

Aamodt, S. M., Kozlowski, M. R., Nordeen, E. J., and Nordeen, K. W. (1992). Distribution and developmental change in [^{3}H] MK-801 binding within zebra finch song nuclei. J. Neurobiol., 23(8), 997-1005.

Adams, M. D., Kelley, J. M., Gocayne, J. D., Dubnick, M., Polymeropoulos, M. H., Xiao, H., Merril, C. R., Wu, A., Olde, B., Moreno, R. F., Kerlavage, A. R., McCombie, W. R., and Venter, J. C. (1991). Complementary DNA sequencing: expressed sequence tags and human genome project. Science, 252, 1651-1656.

Ahmed, S., Kozma, R., Lee, J., Monfries, C., Harden, N., and Lim, L. (1991). The cysteine-rich domain of human proteins, neuronal chimaerin, protein kinase C and diacylglycerol kinase binds zinc: evidence for the involvement of a zinc-dependent structure in phorbol ester binding. Biochem. J., 280, 233-241.

Ahmed, S., Kozma, R., Monfries, C., Hall, C., Lim, H. H., Smith, P., and Lim, L. (1990). Human brain n-chimaerin cDNA encodes a novel phorbol ester receptor. Biochem. J., 272, 767-773.

Alexander, K. A., Cimler, B. M., Meier, K. E., and Storm, D. R. (1987a). Regulation of calmodulin binding to P-57, a neurospecific calmodulin binding protein. J. Biol. Chem., 262, 6108-6113.

Alexander, K. A., Wakim, B. T., Doyle, G. S., Walsh, K. A., and Storm, D. R. (1988). Identification and characterization of the calmodulin-binding domain of neuromodulin, a neurospecific calmodulin-binding protein. J. Biol. Chem., 263, 7544-7549.

Alvarez-Buylla, A., Buskirk, D. R., and Nottebohm, F. (1987). Monoclonal antibody reveals radial glia in adult avian brain. J. Comp. Neurol., 264, 159-170.

Alvarez-Buylla, A.andNottebohm, F. (1988b). Migration of young neurons in adult avian brain. Nature, 335, 353-354.

Anderson, D. J.andAxel, R. (1985). Molecular probes for the development and plasticity of neural crest derivatives. Cell, 42, 649-662.

Anderson, N. G., Maller, J. L., Tonks, N. K., and Sturgill, T. W. (1990). Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. Nature, 343, 651-653.

Apel, E. D., Byford, M. F., Au, D., Walsh, K. A., and Storm, D. R. (1990). Identification of the protein kinase C phosphorylation site in neuromodulin. Biochem., 29, 2330-2335.

Arnold, A. P. (1975). The effects of castration on song development in zebra finches (*Poephila guttata*). J. Exp. Zool., 191, 261-278.

Arnold, A. P. (1980a). Quantitative analysis of sex differences in hormone accumulation in the zebra finch brain: methodological and theoretical issues. J. Comp. Neurol., 189, 421-436.

Arnold, A. P. (1980b). Effects of androgens on volumes of sexually dimorphic brain regions in the zebra finch. Brain Res., 185, 441-444.

Arnold, A. P. (1985a). Gonadal steroid-induced organization and reorganization of neural circuits involved in bird song. In C. W. Cotman (Eds.), Synaptic Plasticity (pp. 263-285). New Nork: Guilford Press.

Arnold, A. P. (1992). Developmental plasticity in neural circuits controlling birdsong: sexual differentiation and the neural basis of learning. J. Neurobiol., 23(10), 1506-1528.

Arnold, A. P., Nottebohm, F., and Pfaff, D. W. (1976). Hormone-concentrating cells in vocal control and other areas of the brain of the zebra finch. J. Comp. Neurol., 165, 487-512.

Ball, G.F. (1990). Chemical neuroanatomical studies of the steroid-sensitive songbird vocal control system: a comparative approach. In: Balthazart J. (Ed.) Hormones, Brain and Behaviour in Vertebrates. 1. Sexual Differentiation, Neuroanatomical Aspects, Neurotransmitters and Neuropeptides. Comp. Physiol vol. 8, Karger, Basel, (pp. 148-167)

Balthazart, J., Foidart, A., Wilson, E. M., and Ball, G. F. (1992). Immunocytochemical localization of androgen receptors in the male songbird and quail brain. J. Comp. Neurol., 317, 407-420.

Barzilai, A., Kennedy, T. E., Sweatt, J. D., and Kandel, E. R. (1989). 5-HT modulates protein synthesis and the expression of specific proteins during long-term facilitation in Aplysia sensory neurons. Neuron, 2, 1577-1586.

Baudier, J., Bronner, C., Kligman, D., and Cole, R. D. (1989). Protein kinase C substrates from bovine brain. J. Biol. Chem., 264(3), 1824-1828.

Baudier, J., Delkoulme, J. C., van Dorsselaer, A., Black, D., and Matthes, H. W. D. (1991a). Purification and characterization of a brain-specific protein kinase C substrate, neurogranin (p17). J. Biol. Chem., 266(1), 229-237.

Baughman, R. W., Huettner, J. E., Jones, K. A., and Khan, A. A. (1991). Cell culture of neocortex and basal forebrain from postnatal rats. In G. Banker & K. Goslin (Eds.), Culturing Nerve Cells (pp. 227-249). Cambridge, MA: MIT Press.

Benowitz, L. (1980). Functional organization of the avian telencephalon. In S. O. E. Ebbesson (Eds.), Comparative neurology of the telencephalon, (pp. 389-421). New York: Plenum.

Bock, E.andDissing, J. (1975). Demonstration of enolase activity connected to the brain-specific protein 14-3-2. Scand. J. Immunol. (Suppl. 2), 4, 31-36.

Boguslawski, G.andPolazzi, J. O. (1987). Complete nucleotide sequence of a gene conferring polymyxin B resistance on yeast: similarity of the predicted polypeptide to protein kinases. Proc. Natl. Acad. Sci. USA, 84, 5848-5852.

Bottjer, S. W., Glaessner, S. L., and Arnold, A. P. (1985). Ontogeny of brain nuclei controlling song learning and behavior in zebra finches. J. Neurosci., 5, 1556-1562.

Bottjer, S. W., Halsema, K. A., Brown, S. A., and Meisner, E. A. (1989). Axonal connections of a forebrain nucleus involved with vocal learning in zebra finches. J. Comp. Neurol., 379, 312-326.

Bottjer, S. W.andJohnson, F. (1992). Matters of life and death in the songbird forebrain. J. Neurobiol., 23(9), 1172-1191.

Bottjer, S. W., Miesner, E. A., and Arnold, A. P. (1984). Forebrain lesions disrupt develpment but not maintenance of song in passerine birds. Science, 224, 901-903.

Branks, P. L.andWilson, M. C. (1986). Patterns of gene expression in the murine brain revealed by in situ hybridization of brain-specific mRNAs. Mol. Brain Res., 1, 1-16.

Braun, K., Scheich, H., Heizmann, C. W., and Hunziker, W. (1991). Parvalbumin and calbindin-D28K immunoreactivity as developmental markers of auditory and vocal motor nuclei of the zebra finch. Neuroscience, 40(3), 853-869.

Brenowitz, E. A. (1991). Evolution of the vocal control system in the avian brain. Sem. Neurosci., 3, 399-407.

Burd, G.andNottebohm, F. (1985). Ultrastructural characterization of synaptic terminals formed on newly generated neurons in a song control nucleus of the adult canary forebrain. J. Comp. Neurol., 240, 143-152.

Chikaraishi, D. M. (1979). Complexity of cytoplasmic polyadenylated and nonpolyadenylated rat brain ribonucleic acids. Biochemistry, 18, 3249-3256.

Cimler, B. M., Andreasen, T. J., Andreasen, K. I., and Storm, D. R. (1985). P-57 is a neural specific calmodulin-binding protein. J. Biol. Chem., 260(19), 10784-10788.

Clayton, D. F.andAlvarez-Buylla, A. (1989). In situ hybridization using PEG-embedded tissue and riboprobes: increased cellular detail coupled with high sensitivity. J. Histochem. Cytochem., 37, 389-393.

Clayton, D. F.andHuecas, M. (1990). Forebrain-enriched RNAs of the canary: a population analysis using hybridization kinetics. Mol. Brain Res., 7, 23-30.

Clayton, D. F., Huecas, M. E., Sinclair-Thompson, E. Y., Nastiuk, K. L., and Nottebohm, F. (1988). Probes for rare mRNAs reveal distributed cell subsets in canary brain. Neuron, 1, 249-261.

Cobb, M. H., Boulton, T. G., and Robbins, D. J. (1991a). Extracellular signal-regulated kinases: ERKs in progress. Cell Regul., 2, 965-978.

Cooper, J. A., Sefton, B. M., and Hunter, T. (1984). Diverse mitogenic agents induce the phosphorylation of two related 42,000 dalton protein on tyrosine in quiescent chick cells. Mol. Cell. Biol., 4, 30-37.

Cowman, A. F., Saint, R. B., Coppel, R. L., Brown, G. V., Anders, R. F., and Kemp, D. J. (1985). Conserved sequences flank variable tandem repeats in two S-antigen genes of Plasmodium falciparum. Cell, 40, 775-783.

Crews, C. M., Alessandrini, A., and Erikson, R. L. (1992). The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. Science, 258, 478-480.

Deloulme, J. C., Sensenbrenner, M., and Baudier, J. (1991). A rapid purification method for neurogranin, a brain specific calmodulin-binding protein kinase C substrate. FEBS, 282(1), 183-188.

DeVoogd, T. J.andNottebohm, F. (1981). Gonadal hormones induce dendritic growth in the adult avian brain. Science, 214, 202-204.

Diekmann, D., Brill, S., Garrett, M. D., Totty, N., Hsuan, J., Monfries, C., Hall, C., Lim, L., and Hall, A. (1991). *Bcr* encodes a GTPase-activating protein for $p21^{rac}$. Nature, 351, 400-402.

Feinberg, A. P.andVogelstein, B. (1984). Addendum: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Bioch., 137, 266-267.

Gahr, M., Guttinger, H.-R., and Kroodsma, D. E. (1993). Estrogen receptors in the avian brain: survey reveals general distribution and forebrain areas unique to songbirds. J. Comp. Neurol., 327, 112-122.

Geschwind, D. H.andHockfield, S. (1989). Identification of proteins that are developmentally regulated during early cerebral corticogenesis in the rat. J. Neurosci., 9(12), 4303-4317.

Goldman, S. A.andNottebohm, F. (1983). Neuronal production, migration and differentiation in a vocal control nucleus of the adult female canary brain. Proc. Natl. Acad. Sci. USA, 80, 2390-2394.

Goslin, K., Schreyer, D. J., Skene, J. H. P., and Banker, G. (1990). Changes in the distribution of GAP-43 during the development of neuronal polarity. J. Neurosci., 10(2), 588-602.

Gubler, U.andHoffman, B. (1983). A simple and very efficient method for generating cDNA libraries. Gene, 25, 263-269.

Hall, C., Monfries, C., Smith, P., Lim, H. H., Kozma, R., Ahmed, S., Vanniasingham, V., Leung, T., and Lim, L. (1990a). Novel human brain cDNA encoding a 34,000 M_r protein n-chimaerin, related to both the regulator domain of protein kinase C and BCR, the product of the breakpoint cluster region gene. J. Mol. Biol., 211, 11-16.

Hanks, S. K., Quinn, A. M., and Hunter, T. (1988). The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science, 241, 42-52.

Harada, J. J., DeLisle, A. J., Baden, C. S., and M.S., C. (1989). Unusual sequence of an abscisic acid-inducible mRNA shich accumulates late in Brassica napus seed development. Plant Mol. Biol., 12, 395-401.

Hariharan, I. K.andAdams, J. M. (1987). cDNA sequence for human *bcr*, the gene that translocates to the *abl* oncogene in chronic myeloid leukemia. EMBO J., 6(1), 115-119.

Harlow, E.andLane, D. P. (1988). Antibodies: a laboratory manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

Heisterkamp, N., Stam, K., Groffen, J., de Klein, A., and Grosveld, G. (1985). Structural organization of the *bcr* gene and its role in the Ph' translocation. Nature, 315, 758-761.

Hendry, S. H. C., Hockfield, S., Jones, E. G., and McKay, R. (1984). Monoclonal antibody that identifies subsets of neurons in the central visual system of monkey and cat. Nature, 307, 267-269.

Herrmann, K.andBischof, H.-J. (1986). Delayed development of song control nuclei in the zebra finch is related to behavioral development. J. Comp. Neurol., 245, 167-175.

Hockfield, S. J.andMcKay, R. (1983). A surface antigen expressed by a subset of neurons in the vertebrate central nervous system. Proc. Natl. Acad. Sci. USA, 80, 5758-5761.

House, C.andKemp, B. E. (1987). Protein kinase C contains a pseudosubstrate prototope in its regulatory domain. Science, 238, 1726-1728.

Hyden, H.andMcEwen, B. S. (1966a). A glial protein specific for the nervous system. Proc. Natl. Acad. Sci. USA, 55, 354-358.

Immelmann, K. (1969). Song development in the Zebra Finch and other estrilid finches. In R. A. Hinde (Eds.), Bird Vocalizations (pp. 61-74). Cambridge, UK: Cambridge University Press.

Kennedy, T. E., Gawinowicz, M. A., Barzilai, A., Kandel, E. R., and Sweatt, J. D. (1988). Sequencing of proteins from two-dimensional gels by using in situ digestion and transfer of peptides to polyvinylidene difluoride membranes: Application to proteins associated with sensitization in Aplysia. Proc. Natl. Acad. Sci., USA, 85, 7008-7012.

Kirn, J. R.andNottebohm, F. (1990). Neuronal clusters: incorporation sites for neurons born in adult canary forebrain. Soc. Neurosci. Abstr., 16, 1249.

Kligman, D.andHilt, D. C. (1988). The S100 protein family. Trends Biochem. Sci., 13, 437-443.

Konishi, M. (1989). Birdsong for neurobiologists. Neuron, 3, 541-549.

Konishi, M.andAkutagawa, E. (1985a). Neuronal growth, atrophy and death in a sexually dimorphic song nucleus in the zebra finch. Nature, 315, 145-147.

Konopka, J. B., Watanabe, S. M., and Witte, O. N. (1984). An alteration of the human c-abl protein in K562 leukaemia cells unmaska associated tyrosine kinase activity. Cell, 37, 1035-1042.

Kosako, H., Gotoh, Y., Matsuda, S., Ishikawa, M., and Nishida, E. (1992). Xenopus MAP kinase activator is a serine/threonine/tyrosine kinase activated by threonine phosphorylation. EMBO J., 11, 2903-2908.

Kozak, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell, 44, 283-292.

Kroodsma, D.andBaylis, J. R. (1982). A world survey of evidence for vocal learning. In D. E. Kroodsma & E. H. Miller (Eds.), Acoustic Communication in Birds (pp. 311). New York: Academic Press.

LaBate, M. E.andSkene, J. H. P. (1989). Selective conservation of GAP-43 structure in vertebrate evolution. Neuron, 3, 299-310.

Laemmli, E. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-685.

Levitt, P. (1984). A monoclonal antibody to limbic system neurons. Science, 223, 299-301.

Lifshitz, B., Fainstein, E., Marcelle, C., Shtivelman, E., Amson, R., Gale, R. P., and Canaani, E. (1988). *bcr* genes and transcripts. Oncogene, 2, 113-117.

Malenka, R. C., Kauer, J. A., Perkel, D. J., Mauk, M. D., Kelly, P. T., Nicoll, R. A., and Waxham, M. N. (1989). An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. Nature, 340, 554-556.

Malinow, R., Schulman, H., and Tsien, R. W. (1989). Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. Science, 245, 862-866.

Marangos, P. J., Zomzely-Neurath, C., and York, C. (1975). Isolation and characterization of the nervous system specific protein 14-3-2 from rat brain. J. Biol. Chem., 250, 1884-1901.

Marler, P., Peters, S., Ball, G. F., Dufty Jr., A. M., and Wingfield, J. C. (1988). The role of sex steroids in the acquisition and production of birdsong. Nature, 336, 770-772.

Mello, C. V., Vicario, D. S., and Clayton, D. F. (1992). Song presentation induces gene expression in the songbird forebrain. Proc. Natl. Acad. Sci., 89, 6818-6822.

McCasland, J. S. (1987). Neuronal control of bird song production. J. Neurosci., 7, 23-39.

McKay, R. G. D.andHockfield, S. J. (1982). Monoclonal antibodies distingish antigenically discrete neuronal types in the vertebrate central nervous system. Proc. Natl. Acad. Sci. USA, 79, 6747-6741.

Milner, R. J.andSutcliffe, J. G. (1983a). Gene expression in rat brain. Nucl. Acids Res., 11(16), 5497-5520.

Mooney, R. (1992). Synaptic basis for developmental plasticity in a birdsong nucleus. J. Neurosci., 12(7), 2464-2477.

Moore, B. W. (1965b). A soluble protein characteristic of the nervous system. Biochem. Biophys. Res. Commun., 19, 739-.

Moore, B. W.andMcGregor, D. (1965a). Chromatographic and electrophoretic fractionation of soluble proteins of brain and liver. J. Biol. Chem., 240, 1647-1653.

Morrison, R. G.andNottebohm, F. (1990). Role of LMAN in post-critical period song learning in zebra finches. Soc. Neurosci. Abstr., 16(449.5), 1099.

Nadin-Davis, S. A.andNasim, A. (1988). A gene which encodes a predicted protein kinase can restore some functions of the ras gene in fission yeast. EMBO J., 7, 985-993.

Nakielny, S., Cohen, P., Wu, J., and Sturgill, T. (1992). MAP-kinase activator from insulin-stimulated skeletal muscle is a protein threonine/tyrosine kinase. EMBO J., 11(6), 2123-2129.

Nei, M. (1987). Molecular evolutionary genetics. New York: Columbia University Press.

Nishizuka, Y. (1988). The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature, 334, 661-665.

Nordeen, E. J.andNordeen, K. W. (1988a). Sex and regional differences in the incorporation of neurons born during song learning in zebra finches. J. Neurosci., 8, 2869-2874.

Northcutt, R. G. (1981). Evolution of the telencephalon in nonmammals. Ann. Rev. Neurosci., 4, 301-350.

Nottebohm, F. (1980). Testosterone triggers growth of brain vocal control nuclei in adult female canaries. Brain Res., 189, 429-436.

Nottebohm, F. (1981a). A brain for all seasons: cyclical anatomical changes in song control nuclei of the canary brain. Science, 214, 1368-1370.

Nottebohm, F., Kelley, D. B., and Paton, J. A. (1982). Connections of vocal control nuclei in the canary telencephalon. J. Comp. Neurol., 207, 344-357.

Nottebohm, F. (1987a). Plasticity in adult avian central nervous system: possible relation between hormones, learning and brain repair. In V. Mountcastle (Eds.), Handbook of Physiology, Section 1:

Higher functions of the nervous system. (pp. 85-108). Bethesda, MD: Am. Physiological Society.

Nottebohm, F., Nottebohm, M. E., and Crane, L. (1986). Developmental and seasonal changes in canary song and their relation to changes in the anatomy of song-control nuclei. Behav. Neural Biol., 46, 445-471.

Nottebohm, F., Stokes, T., and Leonard, C. M. (1976a). Central control of song in the canary. J. Comp. Neurol., 165, 457-486.

Olds, J. L., Anderson, M. L., McPhie, D. L., Staten, L. D., and Alkon, D. (1989). Imaging of memory-specific changes in the distribution of protein kinase C in the hippocampus. Science, 245, 866-869.

Ono, Y., Fujii, T., Igarashi, K., Kuno, T., Tanaka, c., Kikkawa, U., and Nishizuka, Y. (1989). Phorbol ester binding to protein kinase C requires a cysteine-rich zinc-finger-like sequence. Proc. Natl. Acad. Sci. USA, 86, 4868-4871.

Oyler, G. A., Higgins, G. A., Hart, R. A., Battenberg, E., Billingsley, M., Bloom, F. E., and Wilson, M. C. (1989). The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. J. Cell Biol., 109(6:1), 3039-3052.

Paton, J. A.andNottebohm, F. N. (1984). Neurons generated in the adult avian brain are recruited into functional circuits. Science, 225, 1046-1048.

Pearson, W. R.andLipman, D. J. (1988). Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. U.S.A., 85, 2444-2448.

Pickel, V. M., Reis, D. J., Marangos, P. J., and Zomzely-Neurath, C. (1976). Immunocytochemical localization of nervous system specific protein (NSP-R) in rat brain. Brain Res., 105, 184-187.

Porteus, M. H., Brice, A. E. J., Bulfone, A., Usdin, T. B., Ciaranello, R. D., and Rubenstein, J. L. R. (1992). Isolation and characterization of a library of cDNA clones that are preferentially expressed in the embryonic telencephalon. Mol. Brain Res., 12, 7-22.

Qju, M.-S.andGreen, S. H. (1992). PC12 cell neuronal differentiation is associated with prolonged $p21^{ras}$ activity and consequent prolonged ERK activity. Neuron, 9, 705-717.

Reiner, A. (1986). Is prefrontal cortex found only in mammals? Trends Neurosci., 9, 298-300.

Represa, A., Deloulme, J. C., Sensenbrenner, M., Ben-Ari, Y., and Baudier, J. (1990). Neurogranin: immunocytochemical localization of a brain-specific protein kinase C substrate. J. Neurosci., 10(12), 3782-3792.

Rogers, A. W. (1979). Techniques in Autoradiography. New York: Elsevier Science Publishing, Inc.

Saini, K. D.andLeppelsack, H.-J. (1981). Cell types of the auditory caudomedial neostriatum of the starling (*Sturnus vulgaris*). J. Comp. Neurol., 198, 209-229.

Scharff, C.andNottebohm, F. (1991). A comparative study of the behavioral deficits following lesions of various parts of the zebra finch song system: implications for vocal learning. J. Neurosci., 11(9), 2896-2913.

Shtivelman, E., Lifshitz, B., Gale, R. P., and Canaani, E. (1985). Fused transcript of *abl* and *bcr* genes in chronic myelogenous leukaemia. Nature, 315, 550-554.

Simon, M., Strathmann, M., and Gautam, N. (1991). Diversity of G proteins in signal transduction. Science, 252, 802-808.

Simpson, H. B.andVicario, D. S. (1991a). Early estrogen treatment alone causes female zebra finches to produce learned, male-like vocalizations. J. Neurobiol., 22(755-776).

Simpson, H. B.andVicario, D. S. (1991b). Early estrogen treatment of female zebra finches masculinizes the brain pathway for learned vocalizations. J. Neurobiol., 22(7), 777-793.

Stokes, T., Leonard, C., and Nottebohm, F. (1974). The telencephalon, diencephalon and mesencephalon of the canary, *Serinus canaria*, in stereotaxic coordinates. J. Comp. Neurol., 156, 337-374.

Sutcliffe, J. G., Milner, R. J., Shinnick, T. M., and Bloom, F. E. (1983b). Identifying the protein products of brain-specific genes with antibodies to chemically synthesized peptides. Cell, 33, 671-682.

Teague, M. A., Chaleff, D. T., and Errede, B. (1986). Nucleotide sequence of the yeast regulatory gene STE7 predicts a protein homologous to protein kinases. Proc. Natl. Acad. Sci. USA, 83, 7371-7375.

Timmons, M. S.andWitte, O. N. (1989). Structural characterization of the BCR gene product. Oncogene, 4, 559-567.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. U.S.A., 76, 4350-4354.

Watson, J. B., Battenberg, E. F., Wong, K. K., Bloom, F. E., and Sutcliffe, J. G. (1990). Subtractive cDNA cloning of RC3, a rodent cortex-enriched mRNA encoding a novel 78 residue protein. J. Neurosci. Res., 26, 397-408.

Wendland, B., Miller, K. G., Schilling, J., and Scheller, R. H. (1991). Differential expression of the p65 gene family. Neuron, 6, 993-1007.

Wu, J., Michel, H., Rossomando, A., Haystead, T., Shabanowitz, J., Hunt, D. F., and Sturgill, T. W. (1992a). Renaturation and partial peptide sequencing of mitogen-activated protein kinase (MAP kinase) activator from rabbit skeletal muscle. Biochem. J., 285, 701-705.

End