Gene Expression in Brain

edited by

CLAIRE ZOMZELY-NEURATH

and

WILLIAM A. WALKER

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Preface

Gene expression per se has been studied extensively during the past two decades. The advances made in recombinant DNA technology have led to discoveries in molecular genetics that have revealed much about the basic mechanisms on which life depends. However, if one wants to approach complex functions, and the most complex and potentially most interesting is that of brain, one has no choice but to investigate this organ. Until recently molecular approaches to the analysis of the nervous system have only been utilized by a few investigators. This is not surprising since the brain is a complex system and has been, to a large extent, inaccessible to the application of the techniques used so effectively by molecular biologists in their investigations of simpler bacterial and eukaryotic organisms. However, the recent developments in recombinant DNA research and related methods now make it possible to study complex biological problems. Indeed, the area of molecular neurobiology is now being pursued actively by molecular biologists as well as neuroscientists using the powerful tools developed by the former. The coupling of the exciting developments in molecular biology and neuroscience should have a powerful impact not only on basic neuroscience but also on clinical medicine.

The approaches used by investigators in the area of molecular neurobiology are presented in this volume. A range of options exists for the methods used to obtain DNA for amplification by cloning. Some of these include the use of synthetic oligonucleotide probes as, for example, in the chapters on pro-opiomelanocortin, myelin basic protein, and vasoactive intestinal polypeptide (also present in brain), synthesis of complementary DNA (cDNA) from messenger RNAs (mRNA) present in relatively high abundance (tubulin and actin) to mRNA representing less than 1% of the total mRNA population (phenylethanolamine *N*-methyl transferase [PNMT]). The scope of the chapters in this volume ranges from studies on the bag cell neurons of *Aplysia* to the molecular biology of human brain showing that a variety of

approaches can yield important information on genetic expression in the nervous system. The authors have all combined cellular and molecular biology in the research presented in these chapters.

The exciting area of molecular approaches to the study of gene expression in brain has enormous research potential and should make this volume of interest not only to neuroscientists but also to molecular biologists contemplating the study of molecular genetics in complex systems such as the brain. The purpose of this volume is to show what has been accomplished, the various approaches that can be used, and to promote research efforts in this area.

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Honolulu, Hawaii
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The Construction and Identification of Recombinant DNA Probes for the Study of Gene Expression in Nervous Tissue

Barry B. Kaplan, Anthony E. Gioio, and David K. Batter

The complexity of structure and function in the mammalian nervous system has, through the years, necessitated a multidisciplinary experimental approach to its study. Recently nucleic acid hybridization analysis of brain RNA populations has revealed that the number of different genes expressed in brain far exceeds that transcribed in other somatic tissues and organs. The remarkable diversity of gene expression in brain bears on many issues in neurobiology, including the degree to which this large amount of genetic information participates in the development and maintenance of brain structure and function. The findings of RNA-DNA hybridization studies with regard to the diversity of gene expression in different brain regions, neural cell types, and during development and aging have been reviewed (Chaudhari and Hahn, 1983; Dokas, 1983; Kaplan and Finch, 1982). While this approach can provide new insights into gene expression in brain, it is not in itself entirely satisfactory. The complexity of brain RNA populations, coupled with limits in the resolution of the hybridization "assay," preclude one from addressing many important questions concerning the regulation of gene expression in nervous tissue.

The advent of recombinant DNA technology has opened several new avenues of approach to the study of neural gene activity. In this chapter we describe strategies and procedures involved in the construction of cloned complementary DNA (cDNA) probes for specific proteins of neurobiological interest. To illustrate the types of results achieved, data will be given for the cloning of DNA complementary to mRNA coding for phenylethanolamine *N*-methyltransferase (PNMT), the final enzyme in the catecholamine biosynthetic pathway. The utility of cloned cDNA probes will also be discussed.

1. ISOLATION OF SPECIFIC mRNA

To date, detailed study of gene structure and regulation has focused on those systems which produce particular mRNAs in high abundance (i.e., a large number of copies per cell). Consequently, much of our present knowledge of eukaryotic gene expression is derived from analysis of mRNAs coding for highly specialized cell products. Outstanding examples are actin (Engel et al., 1982; Schwartz et al., 1980), albumin (Sala-Trepet et al., 1979; Gordon et al., 1978), amylase (MacDonald et al., 1980), fibrinogen (Crabtree and Kant, 1981), ovalbumin (Breathnach et al., 1977; Dugaiczyk et al., 1978; McReynolds et al., 1978; Roop et al., 1978), ovomucoid (Buell et al., 1979; Nordstrom et al., 1979), conalbumin (Lee et al., 1980), casein and α-lactalbumin (Richards et al., 1981), globin (Efstratiadis et al., 1977; Kinniburgh et al., 1978; Tilghman et al., 1978), immunoglobulins (Adams et al., 1980; Gough et al., 1980; Schibler et al., 1978), vimentin (Zehner and Paterson, 1983),

and vitellogenin (Ohno et al., 1980; Smith et al., 1979; Wahli et al., 1978). However, due to the heterogeneity of cell types comprising nervous tissue and the striking complexity of the mRNA population, mRNAs for brain-specific proteins will frequently be present in lower abundance. Therefore, the levels of these mRNAs will often require enrichment to facilitate the construction and identification of recombinant plasmids. One experimental approach to this problem is outlined below.

1.1. Polyribosome Preparation and Immunopurification

Recent refinements in recombinant DNA technology obviate the need to obtain specific mRNAs purified to homogeneity. Rather, one can use cloning procedures to select the desired recombinant plasmid from a cDNA library constructed from a poly(A+) mRNA population simply enriched for the appropriate mRNA.

Figure 1 outlines an immunological approach to the enrichment of specific mRNA. The method exploits the ability of antibodies raised against native protein to react with the corresponding nascent polypeptide chain on the polyribosome. The soluble antibody–polyribosome complex is subsequently isolated by binding to protein A–Sepharose (Schutz et al., 1977; Shapiro and Young, 1981). Nonreacted polyribosomes are removed by extensive washing, and immunoadsorbed mRNA eluted by disruption of polyribosomes with EDTA. Poly(A+) mRNA is then isolated from total polysomal RNA by affinity chromatography on oligo (dT)-cellulose.

The assumptions inherent in the strategy outlined in Figure 1 are threefold. First, that polyribosomes isolated by standard procedures contain intact nascent peptide chains. Second, that polyribosome integrity can be maintained throughout the extensive exposure to antibody and chromatographic fractionation required by the enrichment procedure. Finally, that nascent peptide chains contain a sufficient number of antigenic determinants to efficiently react with antibody. The validity of these assumptions was investigated as described below.

Data in Figure 2 demonstrate that polyribosomes isolated by sedimentation through 2.0 *M* sucrose contain nascent chains. To prevent ribosome "run-off," polyribosomes were prepared in the presence of cycloheximide, an inhibitor of chain elongation. In this experiment, approximately 90% of the radiolabeled amino acid was associated with polyribosomes sedimenting at > 200 S.

Exposure of polyribosomes to antibody with subsequent chromatographic fractionation had no deleterious effect on polyribosome integrity as shown in Figure 3. Here, protein A–Sepharose chromatography was also used to remove trace ribonuclease (RNase) activity from an IgG fraction prepared from

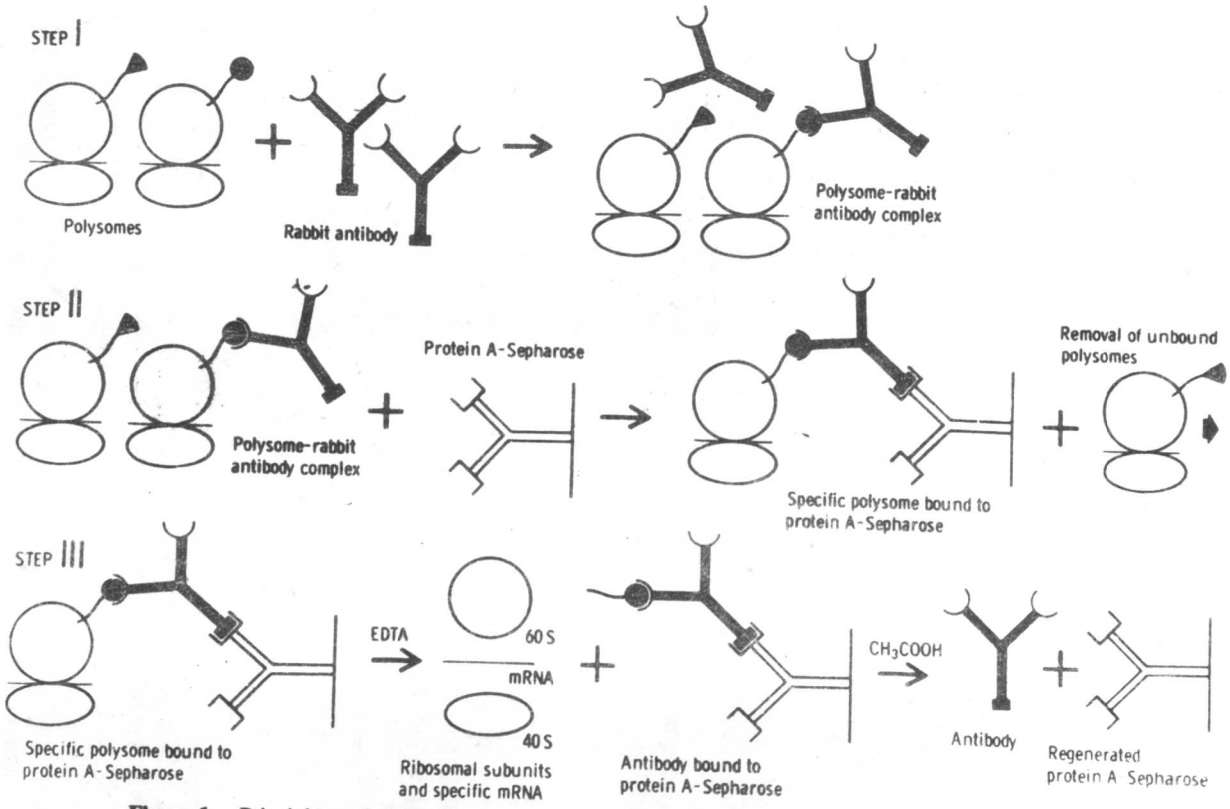

Figure 1. Principles underlying the immunopurification of specific mRNAs. Modified from Schütz et al., 1977.

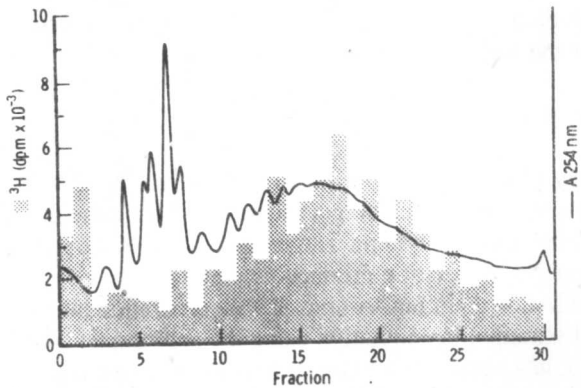

Figure 2. Sucrose density gradient profile of total pulse-labeled pheochromocytoma (PC-12) cell polyribosomes. PC-12 cells were grown in the presence of a [^{3}H]-labeled amino acid mixture (5 min) and polyribosomes isolated from a detergent-treated postmitochondrial supernatant by centrifugation through 2.0 *M* sucrose (Baetge et al., 1981). All solutions used in polysome isolation contained a 1000-fold excess of unlabeled amino acid and cycloheximide (2 μg/mL). Polyribosomes were resuspended and centrifuged through linear 15–45% sucrose gradients in a SW-41 rotor at 36,000 rpm for 75 min at 2°C. Migration is from left to right.

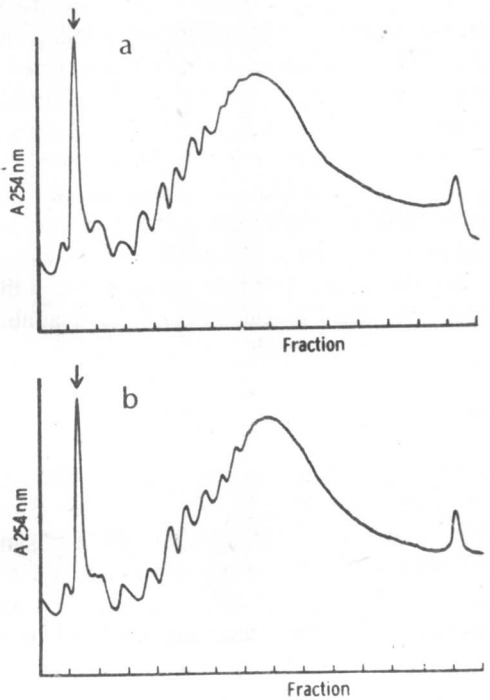

Figure 3. Sucrose density gradient profile of polyribosomes before (a) and after (b) immunoadsorption. Polyribosomes prepared from bovine adrenal medulla were reacted with protein A–Sepharose purified rabbit antibodies raised against PNMT (10 μg IgG/15 A_{260} units of polyribosomes) for 2 hr at 4°C and subsequently passed over a protein A–Sepharose column (Shapiro and Young, 1981). Polyribosomes were displayed on linear sucrose gradients as described in Figure 2. Arrow indicates the position of the 80S monomer. Migration is from left to right.

monospecific rabbit antisera. The quality of the polyribosomes recovered in the column eluent was surprising, providing one an opportunity to reuse the sample for the isolation of a second mRNA (see below).

1.2. mRNA Translation

The efficacy of the immunopurification procedure is most frequently evaluated by *in vitro* translation using a micrococcal nuclease-treated reticulocyte or wheat germ cell-free system. The incorporation of radiolabeled amino acid into total mRNA translation products is monitored by acid precipitation and liquid scintillation counting. Amino acid incorporation into specific protein is estimated by immunoprecipitation using the appropriate antibody. The interpretation of data obtained from the cell-free translation assay assumes that all mRNAs are equally efficient in directing protein synthesis in a heterologous system and that the immunoprecipitation of specific translation products is quantitative.

The level of enrichment achieved by immunoadsorption of bovine adrenomedullary polyribosomes using rabbit monospecific antiserum prepared against cow adrenal PNMT (Park et al., 1982) has been evaluated. Data in Table 1 indicate that PNMT antibody immunoprecipitates 0.6% of the total radioactivity incorporated into acid-insoluble products by a cell-free system programmed with bovine adrenomedullary total mRNA. In comparison PNMT comprised approximately 30% of the translation products synthesized from PNMT-enriched mRNA, a 50-fold enrichment. In this experiment, polyribosomes that did not bind to protein A–Sepharose when challenged with PNMT antibody were recovered, reacted with antibodies raised against tyrosine hydroxylase (TH), and repassed over protein A–Sepharose (see Section 1.1). Data obtained from the *in vitro* translation of control and TH-enriched mRNA are also given in Table 1. The reaction of polyribosomes with TH antibody resulted in a 32-fold elevation of TH-mRNA levels. Based upon the control data in Table 1 and the levels of enrichment achieved in the immunoadsorbed mRNA, we estimate that 38 and 28% of the available PNMT- and TH-mRNA was recovered, respectively.

1.3. RNA-DNA Hybridization

The efficiency of the mRNA enrichment procedure can also be evaluated independently by RNA-DNA hybridization analysis. In this study complementary DNA of high specific activity (2×10^8 dpm/μg DNA) is synthesized from total mRNA and mRNA enriched for a specific message *in vitro* using a viral reverse transcriptase (see Section 2.1). The cDNA probes are subsequently hybridized back to excesses of their template RNA. Hybridization is monitored by the formation of radiolabeled structures resistant to

TABLE 1. CELL-FREE TRANSLATION OF BOVINE ADRENAL POLY(A+) mRNA[a]

RNA Sample	Total ^{3}H Incorporation[b] (dpm/rx)	TH Incorporation[c] (dpm/rx % total dpm)		PMNT Incorporation[c] (dpm/rx % total dpm)		Enrichment (-fold)
Control mRNA[d]	242,400	750	0.25	1,450	0.60	—
PMNT-enriched mRNA[d]	170,000	—	—	51,500	30.3	50.5
TH-enriched mRNA	296,800	23,600	8.0	—	—	32.0

[a]mRNA was translated in a cell-free system derived from rabbit recticulocytes as previously described (Baetge et. al., 1981) using leucine as radiolabeled amino acid.
[b]dpm corrected for ^{3}H incorporated in control reactions containing no exogenous RNA.
[c]dpm corrected for ^{3}H activity immunoprecipitated by IGg obtained from preimmune serum.
[d]data from Baetge et al. (1983).

hydrolysis by S1 nuclease, a single-strand-specific nuclease prepared from *Aspergillus oryzae*. Under conditions of RNA excess, the hybridization reaction follows pseudo-first-order kinetics. Thus, the larger the RNA sequence complexity (i.e., the greater the number of different mRNA species), the slower the reaction and the longer it takes to reach completion (for review see Kaplan, 1982).

Results of a typical RNA-cDNA hybridization experiment are given in Figure 4. The reaction of total cDNA to its template total adrenomedullary mRNA was relatively slow, spanning approximately five log orders of Cot (Fig. 4b). In contrast, cDNA synthesized from PNMT-enriched mRNA hybridized to its template RNA 100- to 1000-fold faster. According to ideal pseudo-first-order kinetics, the reaction of a single RNA species to its cDNA will reach completion within 1.5 log units of Cot [concentration of nucleic-acid (moles nucleotide per liter) × the time of incubation (sec)]. Data in Figure 4a show an inflection point in the hybridization kinetics of PNMT-cDNA 1.5 log units after initiation of the reaction. This kinetic component

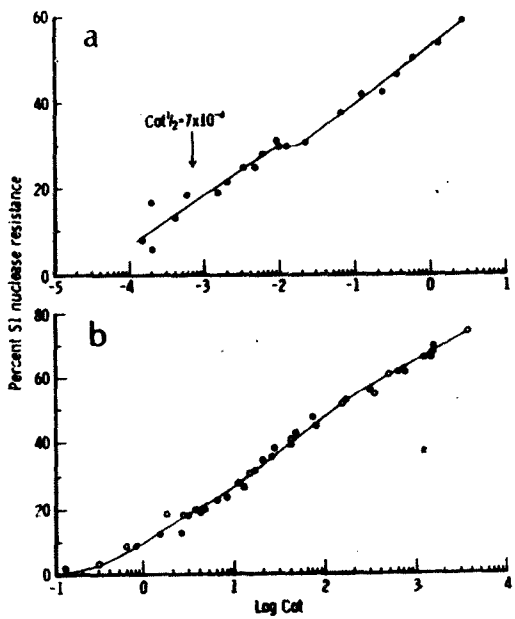

Figure 4. Kinetics of hybridization of cDNA to its template mRNA. [^{32}P]-labeled cDNA was hybridized back to its corresponding template polysomal poly(A+) mRNA prepared from bovine adrenal medulla. RNA concentrations ranged from 0.7 to 10.0 μg/mL. Reactions were carried out in 0.18 *M* Na^+ at 60°C and hybridization monitored by a S1 nuclease/DEAE filter disc assay (Maxwell et al., 1978). (a) cDNA to PNMT-enriched mRNA; (b) cDNA to total mRNA.

comprises approximately 25–30% of the cDNA mass ($Cot_{1/2} \sim 7 \times 10^{-4} M$ sec), a finding which agrees well with the *in vitro* translation data (see Section 1.2).

1.4. Discussion

The experimental approach outlined in Figure 1 permits the isolation of specific mRNAs that have no unusual physicochemical properties to exploit (e.g., size, base composition, or abundance). The efficacy of the approach lies in the use of protein A–Sepharose chromatography which obviates the need to immunoprecipitate the soluble antibody–polyribosome complex with a second antibody, thereby avoiding increased non-specific binding and physical trapping of polyribosomes in the precipitated complex. Additionally, the binding of the antibody–polyribosome complex to protein A–Sepharose facilitates the removal of contaminating polyribosomes by extensive washing of the adsorbed material. The IgG fraction of monospecific antisera, affinity-purified polyclonal antibodies, and monoclonal antibodies have all been employed successfully in the immunoadsorption of specific polyribosomes.

A significant advantage of this approach is that several different mRNAs can be prepared from a single polyribosome preparation (Section 1.3). Moreover, antibody used in the procedure can be recovered from the column (see Fig. 1) with little loss in reactivity. In addition to the preparation of mRNA enriched for PNMT and TH, this method has been used in the purification of mRNA for cystathione synthetase (Kraus and Rosenberg, 1982) and the heavy chain of the human histocompatibility antigen DR (Korman et al., 1982). These proteins are thought to comprise approximately 0.01–0.05% of the total protein population. It seems likely, therefore, that the method described will prove applicable to the purification of other moderately abundant mRNAs from brain.

2. CLONING OF SPECIFIC cDNA

2.1. Synthesis of Double-Stranded cDNA

A schematic diagram outlining the procedures employed in the construction of recombinant plasmids is given in Figure 5. Experimental protocols for each of the procedures illustrated have been well described (Maniatis et al., 1982; Schrader and O'Malley, 1982). Initially, single-stranded cDNA is synthesized from poly(A+) mRNA using a viral reverse transcriptase, deoxyribonucleotides as substrates, and oligo$(dT)_{10-18}$ as a primer [see Fig. 5 (1)].