

Results

A clear 369 bp expected band for Ig light chain cDNA fragment with the cloning site (Fig. 2) and a 390 bp expected band for Ig heavy chain cDNA fragment (Fig. 3) were obtained. The PCR products were subcloned into M13 followed by subsequent sequencing.²⁰ The cDNA cloning of the variable regions of heavy and light chains, were obtained by constructing a conventional cDNA library, has been published elsewhere.¹¹ These cDNA clones were also sequenced.

No mutations were found in the sequences obtained by either the cDNA clone from the conventional cDNA library or from the cDNA amplified fragments produced directly by the PCR of the hybridoma cell RNA.

We are presently developing a method to synthesize chimeric mouse/human monoclonal antibodies by using synthetic long oligomers ligated together containing just the mouse CDRs (complementarity-determining regions), and to synthesize mouse/human MAb containing fewer mouse sequences.

Acknowledgments

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²⁰ J. Vieira and J. Messing, *Gene* **19**, 259 (1982).

[9] Complementary DNA Synthesis *in Situ*: Methods and Applications

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Synthesis of complementary DNA (cDNA) has traditionally been performed on RNA isolated from large amounts of cells or tissue. cDNA probes and cDNA libraries have been successfully constructed from purified RNA; however, the use of isolated RNA from a large tissue source results in the loss of cellular resolution. This is an important consideration given that some RNAs are confined to distinct cell types within a specific tissue. In such situations, the RNA from these few

cells represents only a small percentage of the whole-tissue total RNA. Therefore, by using a tissue as a source for RNA isolation, specific low-abundance RNAs from a few cells will be diluted by the RNA from the surrounding cells. The key to isolation of these specific mRNAs is to enrich for the cells that express the mRNAs. Enrichment schemes encompass many technologies, including fluorescence-activated cell sorting and panning procedures. Alternatively it is possible to enrich for these cells capitalizing on knowledge of their anatomical localization. This can be accomplished by performing cDNA synthesis *in situ*, incorporating radioactivity into the cDNA, and examining the autoradiographic distribution of the resultant radiolabeled cDNA. Such a procedure involving cDNA synthesis *in situ* has been developed and is referred to as *in situ* transcription (IST).¹

In situ transcription was originally used as an mRNA localization technique. Briefly, IST is performed by annealing a specific oligonucleotide primer to the mRNA in a tissue section, washing away the unhybridized primer, and initiating cDNA synthesis by addition of reverse transcriptase and deoxynucleotide triphosphates. This is followed by washing away of the unincorporated triphosphates and autoradiographic exposure of the tissue section. The ability to synthesize cDNA *in situ* allows radiolabeled deoxynucleotides to be incorporated into the growing cDNA molecule in an anatomically defined region. This technology has several applications, including cellular localization of specific mRNAs, assessing transcriptional regulation by pharmacological agents, expression profiling of mRNA populations from anatomically defined areas, cloning of mRNA populations, determining the degree of translational control of specific mRNAs, and analysis of gene expression in live cells. Of the forementioned IST applications, the only topic that will not be discussed extensively in this chapter is the use of IST in cellular localization studies, which has been covered in detail elsewhere.² Each of the other applications will be presented in the context of a specific experimental system. We also present the coupling of IST to a nucleic acid amplification procedure, antisense RNA amplification (aRNA), which facilitates the cloning of mRNA populations from tissue sections as well as the mRNA from a single cell. Many of the procedures for these different topics share procedural steps, therefore repeated reference to these common elements will be apparent in this chapter.

¹ L. H. Tecott, J. D. Barchas, and J. H. Eberwine, *Science* **240**, 1661 (1988).

² I. Zangger, L. Tecott, and J. Eberwine, *Technique* **1**, 108 (1989).

In Situ Transcription in Study of Gene Expression in Developing Mouse Embryos

In Situ Transcription

Developmental biology constitutes one of the most exciting and prolific areas of life science research currently conducted worldwide. As in many research disciplines, advances in the understanding and appreciation of gene expression in developing organisms has been limited by the availability of sufficient quantities of desirable embryonic material from which to isolate mRNA and to develop cDNA libraries to screen. Such limitations have been overcome by the application of *in situ* transcription coupled with aRNA amplification technology (discussed below) to the study of gene expression in mouse embryos during the period of neural tube closure. Specifically, we were interested in determining the relative ratio of specific mRNAs following embryonic exposure to maternal treatments that have previously been shown to disrupt neural tube morphogenesis and result in the production of lethal congenital defects of the nervous system.^{3,4} The following section details the necessary procedural considerations for this type of application.

At the desired gestational stage, the dam is killed and the uterus is removed into a petri dish containing ice-cold Krebs buffer. Starting at the ovarian end, the decidual capsule is torn open with watchmaker's forceps under a dissecting microscope. The embryos are then carefully dissected free from their extraembryonic membranes, with extreme care taken not to prematurely "pop" the embryo out of the chorionic sac. Those embryos that remain intact following removal of the amnion are prepared for histological sectioning. We have successfully utilized two different fixation protocols. The first procedure involved placing the embryos for at least 48 hr in Bodian's fixative (80% ethanol, 37% formaldehyde, and 5% glacial acetic acid). Similarly, we have also used 4% paraformaldehyde as a fixative (for 5 min) with excellent results, noting however that paraformaldehyde-fixed tissue is more difficult to work with and the integrity of the tissue can be compromised at any washes above room temperature. This is in contrast to adult tissue, in which paraformaldehyde fixation is often the method of choice. Once fixed, the embryos are lightly stained with Fast Green⁵ to facilitate orientation of the embryos for sectioning. This staining procedure does not interfere with the cDNA synthesis, and is

³ R. H. Finnell, S. P. Moon, L. C. Abbott, J. A. Golden, and G. F. Chernoff, *Teratology* **33**, 247 (1986).

⁴ R. H. Finnell, G. D. Bennett, S. B. Karras, and V. K. Mohl, *Teratology* **38**, 313 (1988).

⁵ D. R. Hilbelink and S. Kaplan, *Stain Technol.* **53**, 261 (1978).

quite useful for discriminating regions of the embryo for localization experiments. After standard paraffin embedding, 8- μ m-thick sagittal sections of the embryos are cut, mounting three sections on a slide. The slides are then stored in slide boxes at -80° to prevent nucleic acid degradation.

Prior to performing *in situ* transcription on the embryonic slides, it is necessary to remove the paraffin from the sections. This procedure involves several washes to remove the paraffin, followed by a postfixation step. The slides are placed in Coplin jars and initially washed with xylene three times, replacing the solvent every 5 min. The sections are then slowly rehydrated in ethanol, with two 5-min washes of 100, 90, and finally 70% ethanol. The remaining ethanol is washed off with sterile water. The slides should next be washed twice in a phosphate-buffered saline (PBS), changing the PBS after 5 min. After two 5-min fixation steps in 4% paraformaldehyde, the PBS washes are repeated and the slides are then allowed to dry completely, at which point they may be restored at -80° , or directly used for *in situ* transcription studies.

The first step in performing *in situ* transcription from fixed tissue (embryo) sections is the prehybridization step. The slides used for the study must be at room temperature and completely dry. The individual embryo sections are encircled with a ring of rubber cement to create a well. The size of the well is important, for the larger it is the greater the volume of hybridization buffer needed to fill the well, thus increasing the cost. The rubber cement should therefore be as close to the section as possible, without covering any portion of it. This process can easily be accomplished using a 5-ml syringe and a large gauge (16-gauge) needle. The application of the rubber cement is repeated two to three times to create a well of the appropriate depth. Once the rubber cement has dried, the prehybridization buffer can be applied. This buffer is composed of 25% formamide in $5 \times$ standard sodium citrate (SSC). The slides are placed on prehybridization buffer-moistened 3-mm paper in a petri dish or other humidified chamber. The appropriate volume of hybridization buffer is added to cover the section. In our embryonic studies, anywhere from 18 to 25 μ l has been sufficient. With a typical slide containing three embryo sections, two will receive 100 ng of the primer. In these studies a modified oligonucleotide primer that will hybridize to all of the poly(A)⁺ RNA within the cell was chosen. This oligonucleotide was modified so that it can direct the amplification of the cDNA. The oligonucleotide primer is an oligo(dT₂₄)-T7 amplification oligonucleotide, which contains 24 thymidine residues that will hybridize to polyadenylated mRNA molecules. It is important in this procedure to perform the same *in situ* transcription reaction save the addition of the primer on at least one tissue section. This serves as a control for determining the effectiveness of the hybridization

and reverse transcription step, as well as the level of endogenous background activity. The sections are allowed to prehybridize at either room temperature or 37° overnight. For embryonic tissue fixed directly in 4% paraformaldehyde, the prehybridization should be at room temperature.

At the completion of the prehybridization step the buffer is removed from the wells, being careful not to damage the section with the pipette tip. The sections are then placed in Coplin jars and washed in $2 \times$ SSC at room temperature, replacing the fluid after 15 min. The second wash in $0.5 \times$ SSC is for 1 hr and is performed at either 40° or room temperature, depending on the fixative used for the tissue section. At the completion of the washes, the slides must again be allowed to dry completely and the rubber cement should be reapplied to provide wells for the hybridization step. The IST reaction mixture, based on a 50- μ l total volume, is as follows: 5 μ l of $10 \times$ transcription buffer ($1 \times = 50$ mM Tris-HCl, pH 8.3, 6 mM $MgCl_2$, and 0.12 M KCl), 3.75 μ l of 100 μ M dithiothreitol (DTT), 1.25 μ l of dATP (10 mM), 1.25 μ l of dGTP (10 mM), 1.25 μ l of TTP (10 mM), 1 μ l of [α - ^{32} P]dCTP (approximately 800 μ Ci/25 μ l), 4 μ l of reverse transcriptase (at least 20 units/section) and the remaining volume is sterile water (32.5 μ l in this 50- μ l example). No nonlabeled dCTP is added in this reaction in order to enhance the autoradiographic signal. Indeed, one in four nucleotides of the synthesized cDNA will be radiolabeled. The disadvantage of not including nonlabeled dCTP in the reaction is that the cDNA transcripts tend to be shorter (because of low substrate concentration); however, the autoradiographic signal is still specific. Longer cDNA transcripts can be obtained by the addition of nonlabeled dCTP to a final concentration of 25 μ M. For each section, approximately 18–25 μ l of reaction mixture is applied and the humidified petri dish is incubated at 37° for 1 hr. This is best accomplished by floating the dish in a water bath set to the desired temperature. At the completion of the incubation period, the radioactive hybridization buffer is removed from the sections and the slides are rinsed to remove unincorporated deoxynucleotides. The slides can be placed in staining trays that are suspended in a diethyl pyrocarbonate (DEPC)-treated beaker (2000 ml) containing $2 \times$ SSC for the first hour and then $0.5 \times$ SSC for the next 6 to 18 hr. All of the rinses should be at room temperature.

Once the slides have been rinsed and air dried, they are ready to be exposed to X-ray film. Prior to this step, the rubber wells should be removed to permit proper apposition of the sections to the film. The slides can be taped to a cardboard backing and the films can be exposed to the slides either at -80° with an intensifying screen from 5 to 120 min, or at room temperature without a screen for varying lengths of time. Figure 1 represents a typical autoradiographic result of gestational day 10 SWV mouse embryos that were processed for *in situ* transcription. On the left,

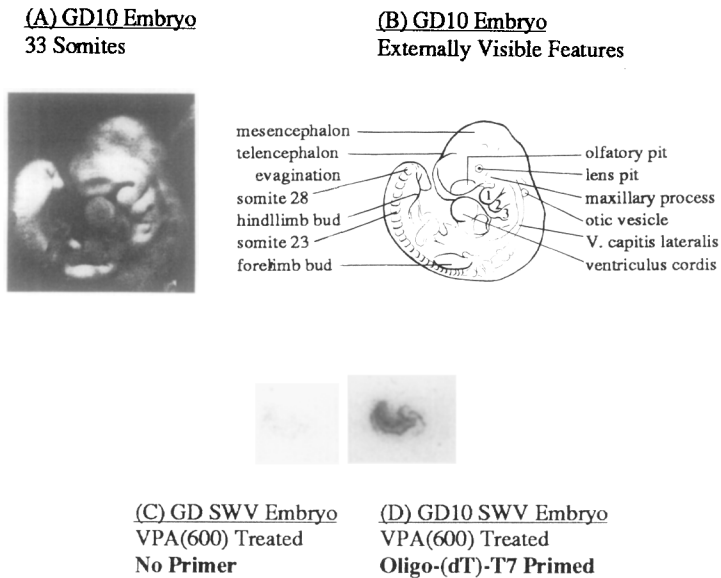


FIG. 1. *In situ* transcription in gestational day (GD) 10 SWV embryos. (a) Photograph and (b) schematic of a GD-10 embryo with approximately 28–35 somite pairs (K. Theiler, “The House Mouse: Atlas of Embryonic Development” Springer-Verlag, New York, 1989). Sections from embryos at this stage of development were taken through the IST procedure. In (c) the section did not receive the oligo(dT)-T7 primer, demonstrating the endogenous level of background binding. In (d) the embryo was hybridized in the presence of the oligo(dT)-T7 primer. Note the intense staining when compared to (c) in areas of rapid cellular proliferation.

where no primer was added to the section, the signal is very faint, indicative of the endogenous background level of hybridization. The embryo on the right was allowed to hybridize in the presence of the oligo(dT)-T7 amplification oligonucleotide primer, which hybridizes to all of the poly(A)⁺ mRNA transcripts. It should be noted that the intense staining, in comparison to the unprimed embryo, occurs most prominently in areas of rapid cellular proliferation, such as the heart and branchial arches.

Amplified Antisense RNA Synthesis

The cDNA that has been synthesized directly on the embryonic section may be removed for further studies by alkaline denaturation of the mRNA–cDNA hybrids that were formed. This is accomplished by carefully adding 20 μ l of 0.5 N NaOH and 0.1% (w/v) sodium dodecyl sulfate (SDS) to each section and repeatedly pipetting up and down until the entire section is removed into a 1.5-ml Eppendorf tube. To this is added 20 μ l of

1 M Tris (pH 7.0) and 1 μ l of tRNA (10 mg/ml) for use as a carrier. After a phenol–chloroform extraction and ethanol precipitation step, the single-stranded cDNA is allowed to self-prime (hairpinning) to initiate its second strand synthesis. This is accomplished by resuspending the dry pellet of cDNA in 10 μ l of water, to which the following are added: 2 μ l of 10 $\times$ React 2 buffer (1 $\times$ = 50 mM Tris, pH 7.0, 50 mM NaCl, 5 mM MgCl₂), 1 μ l each of deoxynucleotides (dATP, dGTP, TTP; 10 mM), 2 μ l of [α -³²P]dCTP, and 1 μ l each of T4 DNA polymerase and Klenow polymerase. This is allowed to incubate for 2 hr at 37°. The next step involves a brief heating at 65° for 3 min to inactivate the DNA polymerase, followed by the addition of 81 μ l of 1 $\times$ S1 nuclease buffer (30 mM sodium acetate, pH 4.5, 50 mM NaCl, 1 mM zinc acetate). One unit of S1 nuclease enzyme is added and the reaction is incubated at 37° for 3 min. Following a phenol–chloroform extraction and ethanol precipitation, the now double-stranded cDNA is ready for blunt ending. To accomplish this, the dry pellet is resuspended in 4.5 μ l of sterile water to which 1 μ l of the following are added: React 2 buffer (10 $\times$), a mix of all four deoxynucleotides (each at 2.5 mM), and 0.5 μ l of T4 DNA polymerase. This mixture is incubated at room temperature for 20 min. At the end of this “filling-in” reaction the sample is phenol–chloroform extracted and ethanol precipitated. The double-stranded cDNA is dissolved in 12 μ l of sterile water followed by drop dialysis against sterile water on a Millipore (Bedford, MA) HAWP filter for no less than 3 hr to remove any unincorporated deoxynucleotides. At this point the double-stranded cDNA is ready to be cloned or amplified for use as a probe.

The amplification of the embryonic cDNA into antisense aRNA can be exceptionally useful in the analysis of gene expression. We have successfully amplified cDNA obtained from *in situ* transcribed cDNA from embryonic tissue sections, essentially creating riboprobes that were used to screen Southern blots containing cDNA clones of interest. To do this, we utilized a Promega (Madison, WI) Riboprobe Gemini System II buffer kit, which provided all of the necessary components for the amplification, except the radiolabeled CTP. In a 20- μ l total volume reaction, the following reagents are utilized: 4 μ l transcription buffer (5 $\times$), 4 μ l of cDNA, 1 μ l of CTP (100 μ M), 1 μ l each of ATP, GTP, and UTP (10 mM), 2 μ l of 0.1 M DTT, 0.5 μ l of RNasin, 1.5 μ l of water, 3 μ l of [α -³²P]CTP, and 1 μ l of concentrated T7 RNA polymerase. Prior to adding the enzyme, 0.5 μ l of the mixture is spotted on a small piece of 1-mm Whatman (Clifton, NJ) paper for scintillation counting. The sample is incubated for 3 hr at 37°, after which a second 0.5- μ l sample is spotted on the Whatman paper, the nucleic acids precipitated in 10% (w/v) trichloroacetic acid (TCA), and the sample counted. At this point the aRNA, which has been amplified

several thousandfold (yet maintains a high degree of fidelity relative to the original mRNA found in the embryo section), can either be used to probe Southern blots or taken through a second round of amplification. The latter involves several procedural steps described elsewhere.^{6,7} After the amplification process is complete, the volume of the aRNA probe is brought up to 60 μ l with sterile water, heat denatured for 5 min, placed on ice for a few minutes, and then applied directly to the blot of interest.

Figure 2 represents the hybridization pattern of an aRNA probe made from a single section of a gestational day 10 SWV mouse embryo. The Southern blot contained 500 ng of the following cDNA clones: Hox 7.0, heat shock protein (HSP) 70, HSP 68, retinoic acid receptor subunits (α , β , and γ), calcium channel, and potassium channel. By quantitating the autoradiographic intensity of the aRNA hybridization to these cDNA clones (taking into account cDNA size and aRNA size), it is possible to measure the relative abundance of the mRNAs of interest. Furthermore, in keeping with our interest in developmental defects, by utilizing embryos that have been exposed *in utero* to various teratogens it is possible to observe fluctuations in gene expression in response to the teratogenic treatment.

In Situ Transcription in Study of Low-Abundance mRNAs from Adult Tissues

Experiments investigating the presence and regulation of mRNAs are critical in understanding behaviors that result from interactions between several cell types. However, these studies when performed *in situ* may be complicated by at least three factors. The relative abundance of different mRNAs varies severalfold and studies of mRNAs of low abundance usually require the use of large amounts of tissue to ensure the isolation of sufficient quantities of these mRNAs. The amount of the mRNAs of interest may be further decreased by dilution from mRNAs present in surrounding cells (as exemplified by the heterogeneity of neurons and glia within the nervous system). Finally, if *in situ* hybridization is the technique of choice for assessment of mRNA regulation it is essential to have prior knowledge of at least a portion of the nucleic acid sequence for the design of nucleotide probes. As previously mentioned, the combination of *in situ*

⁶ J. Eberwine, H. Yeh, K. Miyashiro, Y. Cao, S. Nair, R. Finnell, M. Zettel, and P. Coleman, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3010 (1992).

⁷ J. Eberwine and S. Mackler, in "Molecular Analysis of Cellular Response to Opiate Challenge, Fidia Symposia in Neurosciences" (E. Costa and T. Joh, eds.), (in press), Academic Press, New York, 1992.

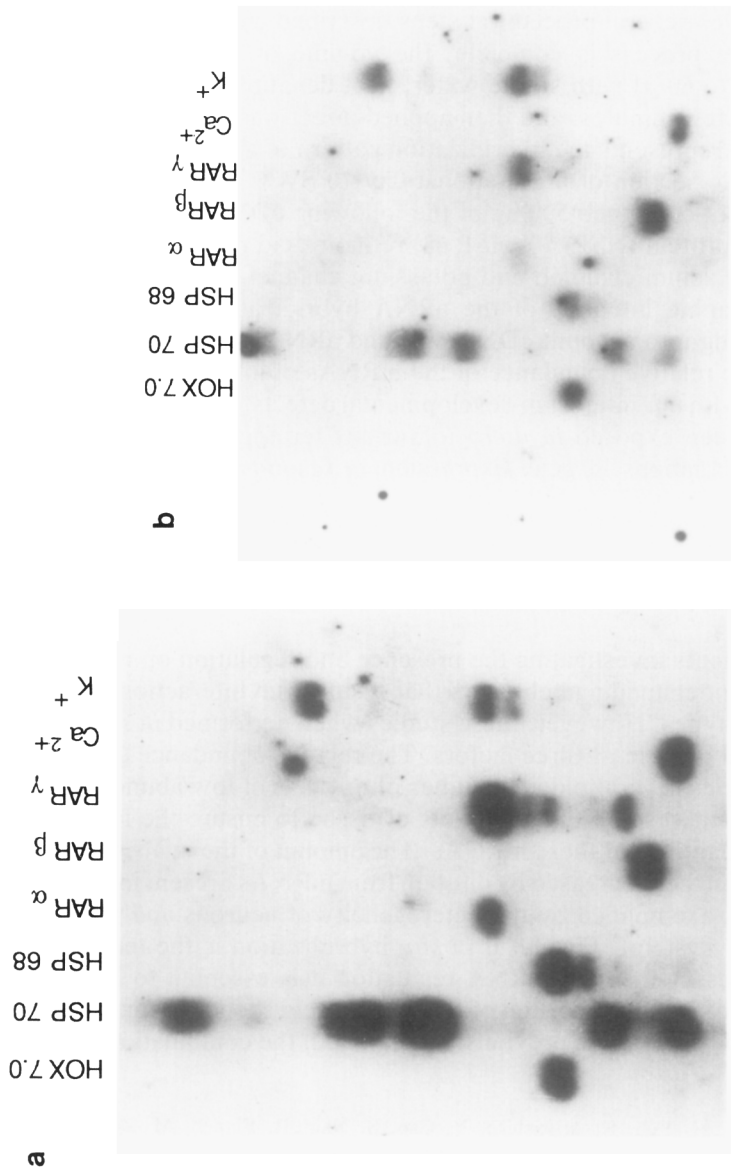


FIG. 2. Genetic expression profiles of GD 10:0 SWV embryos exposed at a critical period of morphogenesis to either a hyperthermic (43°) insult (a) or a 38° control treatment (b). The IST-aRNA-derived probes were hybridized to cDNA clones for Hox 7.0 (homeobox), HSP 70 (heat-shock protein), HSP 68, RAR α (retinoic acid receptor, α subunit), RAR β (β subunit), RAR γ (γ subunit), Ca (calcium channel), and K (potassium channel).

transcription (IST) and antisense RNA amplification (aRNA) has been developed to study both known and novel mRNA molecules in an anatomically defined region of the adult central nervous system (CNS) as well as in tissue culture cell lines. This procedure offers distinct advantages, compared to *in situ* hybridization, and can minimize the aforementioned complicating factors.

In these studies the only significant difference in performing IST on adult tissues, as compared to that described in the previous section for embryonic tissue, is the actual fixation procedure. *In situ* transcription is performed by cutting 10- to 15- μ m-thick sections from fresh frozen tissue (coronal sections including the rat striatum are an example). The sections should be fixed for 5 min in 3% neutral buffered paraformaldehyde followed by several washes in $2\times$ SSC and dehydration through graded ethanols (70, 85, 95, and 100%). These fresh-frozen postfixed sections can be stored at -80° for months to years without significant degradation of the nucleic acids. All solutions should be prepared so that they are RNase free. When ready for use, the sections are slowly brought to room temperature and the tissue is encircled with a thin layer of rubber cement. Surrounding regions of the section that are not important for further study may first be removed with a sterile razor blade. As an example, in studies of the striatum the cerebral cortex can be carefully cut away, leaving the striatum on the slide for IST.

The remainder of the steps in performing IST for this tissue are the same as described in the section *In Situ* Transcription (above). The IST-cDNA can also be removed from the slide and amplified as previously described, or used directly as a probe. We have utilized this procedure to screen for the presence of selected gene families in defined tissue types. Using the rat pituitary as a tissue source, we have annealed a 154-fold degenerate oligonucleotide primer directed against membrane-spanning region 2 of the β -adrenergic receptor to 11- μ m-thick fresh frozen postfixed pituitary sections from rats that had been treated with haloperidol (dopamine receptor antagonist) or bromocriptine (dopamine receptor agonist) (Fig. 3). The cDNA transcripts have been removed and used as a probe to screen Southern blots of genomic DNA. It was assumed that specificity of hybridization would be conferred by the primer and that the IST-cDNAs would be derived from just a few mRNAs that would hybridize to this primer. Indeed, the results presented in this autoradiogram (Fig. 4) show that there are only a few discrete genomic DNA bands hybridizing to the IST-cDNA. While many of the bands were of equal intensity, nonetheless there were other bands whose intensity varied significantly. This suggests that there were differing initial amounts of mRNA corresponding to these genomic DNA bands. It is reasonable to speculate that these cDNAs were

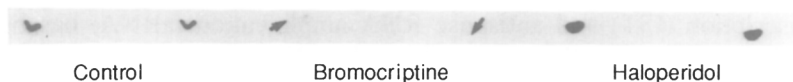


FIG. 3. *In situ* transcription in dopamine-treated rat pituitaries. The IST was performed in rat pituitary sections from animals that were injected with saline (control), bromocriptine (2 mg/kg), or haloperidol (2 mg/kg). Animals were injected once daily for 4 days. The primer in these reactions was directed to the proopiomelanocortin (POMC) mRNA sequence. *In situ* transcription was performed in the presence of radiolabeled dCTP. Exposure of the film autoradiogram was for 10 min. The arrows point to the pituitaries after treatment with bromocriptine, which reduces the amount of POMC mRNA. The increase in signal in the haloperidol-treated animals results from an increase in POMC mRNA.

derived from mRNAs whose abundance was altered by the administered dopaminergic agents. These IST-cDNAs or genomic DNA bands can now be cloned for further characterization.

Electrophoretic Analysis of *in Situ* Transcription-Derived cDNAs and Assessment of Translational Control

For IST to take place, cDNA synthesis must occur. While cDNA synthesis can be detected by autoradiography of the tissue section after incorporation of radiolabeled nucleotides, this analysis does not provide any characterization of the synthesized cDNAs. An analysis of the IST-derived cDNAs was initiated to optimize the reaction conditions, with the rationale that the longer the cDNAs, the greater the signal-to-noise ratio. With this rationale in mind, a strategy was developed for characterization of the cDNA transcripts. The IST-derived cDNAs that are removed from the tissue section (as described in *In Situ* Transcription in Study of Low-Abundance mRNAs from Adult Tissues, above) can be electrophoresed in a denaturing polyacrylamide gel (Fig. 5). The autoradiographic signal obtained from this analysis shows a distinct banding pattern (see Fig. 6). This banding pattern is not generated by alkaline hydrolysis of cDNA because several different denaturing agents, including KOH, NaOH, guanidinium hydrochloride, and guanidinium isothiocyanate, have been used to remove cDNA from the tissue sections. The banding pattern produced from cDNA transcripts is the same for the different denaturants. When proopiomelanocortin (POMC) mRNA from rat and mouse pituitaries is *in situ* transcribed and the transcripts examined by DNA sequencing gel analysis, the banding pattern can be correlated with the sequence of the mRNA. The sequence differences between rat and mouse POMC mRNAs are readily discernible from the gel.⁸ Comparison of the sequence with the bands indicates that the bands correspond primarily with G residues of

⁸ J. Eberwine, D. Newell, C. Spencer, and A. Hoffman, submitted for publication.

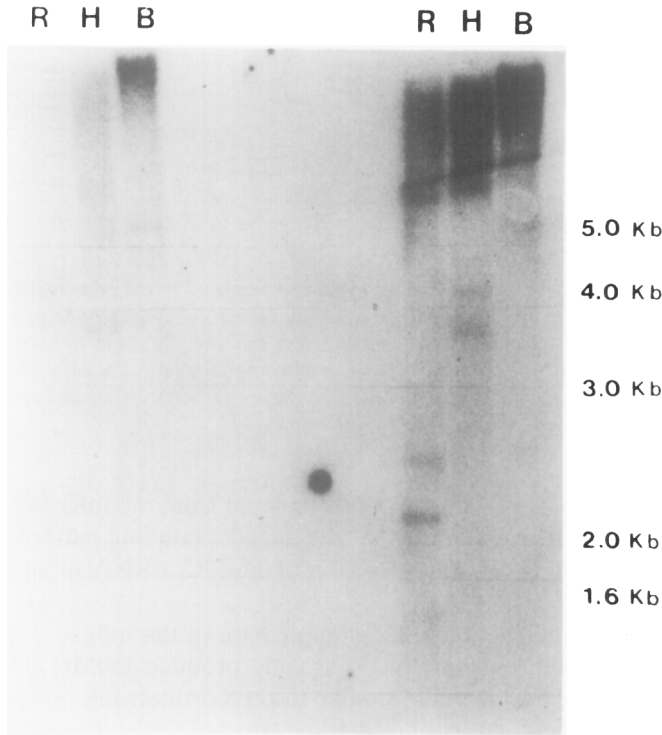


FIG. 4. Rat genomic DNA Southern blot probed with IST-cDNA. Ten micrograms of rat genomic DNA was cut with *EcoRI* (R), *HindIII* (H), or *BamHI* (B). *In situ* transcription was performed on pituitary sections using a degenerate primer directed to the second membrane-spanning region of the G protein-coupled receptors. *Left*: Autoradiogram resulting from probing with IST-cDNA taken from bromocriptine (increases dopamine action)-treated animals. *Right*: Autoradiogram of the haloperidol (HALDOL)-treated animals. The differences in band intensity are reflective of the amount of IST-cDNA in the probe which in turn is reflective of the amount of individual hybridizing mRNAs in the tissue section. (Data were first presented in Eberwine and Mackler,⁸ and is reproduced here with the permission of Academic Press.)

the mRNA and to a lesser extent with A, C, and U. We were initially concerned that these bands resulted from a fixation artifact due to cross-linking of the mRNA to other molecules by the paraformaldehyde treatment of the section.⁹ This is not the case because IST performed on unfixed tissue shows the same banding pattern as that found in fixed tissue (Fig. 6). Additionally, the banding pattern is not a random occurrence due solely to interaction of the primer with the mRNA. We know this because in previous experiments, when primer extension is performed on mRNA in solution, a smear of termination sites is observed; some bands form that

⁹ M. Feldman, *Proc. Nucleic Acid Res. Mol. Biol.* **13**, 1 (1973).

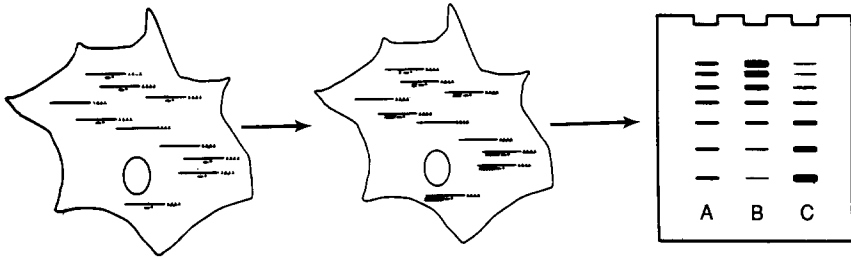


FIG. 5. Use of IST in the analysis of translational control. The experimental methodology of IST includes the *in situ* hybridization of a radiolabeled oligonucleotide primer to fixed cells (left), the production of cDNA *in situ* using IST (middle), and the removal of the transcripts from the section and analysis on a sequencing gel (right). The gel schematic shows the type of autoradiographic intensity shifts that are seen when the translation of an mRNA is decreased (B) or increased (C) relative to control (A).

do not correspond to the same bands isolated from the mRNA in a tissue section (Fig. 6). It is apparent that the specific banding pattern observed for POMC results from an interaction of POMC mRNA *in situ* with the tissue section.

We investigated this banding phenomenon in the mouse AtT-20 pituitary tumor-derived cell line. AtT-20 cells produce POMC mRNA and protein in a regulated manner similar to the corticotroph of the anterior pituitary.¹⁰ This cell line was selected because of the ability to grow large numbers of cells, which provides a constant and consistent source of POMC-producing tissue. For this procedure, AtT-20 cells are plated at an initial density of 1×10^5 cells/well onto sterile poly(L-lysine)-coated glass circular coverslips in 24-well plates. The glass coverslips are first cleaned for 3 min in 0.2 N HCl, followed by 3 min in distilled water and 3 min in acetone, and air dried overnight. The coverslips are then coated in poly(L-lysine) (10 min in 100 mg/500 ml), rinsed in distilled water for 20 sec, and then dried at room temperature for 2 days. The coverslips are rinsed in chloroform in the hood to sterilize them. After 48 hr in culture, the cells are briefly washed twice in phosphate-buffered saline (PBS) at 4°, and then fixed directly in their wells for 20 min in 4% neutral-buffered paraformaldehyde. The cells are then washed briefly in $2 \times$ SSC and stored in their wells for future use in 70% ethanol at 4°. When ready for use, the coverslips should be removed from the wells and allowed to dry. They can then be affixed to microscope slides with rubber cement, which not only facilitates handling but also is useful to form a well around the cells. Approximately 100 μ l of hybridization solution ($4 \times$ SSC, 40% formamide,

¹⁰ V. Hook, S. Helsler, S. Sabol, and J. Axelrod, *BBRC* **106**, 1364 (1982).

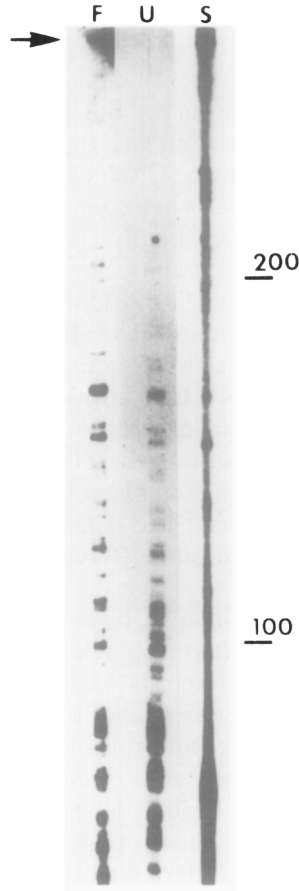


FIG. 6. *In situ* transcription-derived POMC cDNA from rat pituitaries. The POMC IST-cDNA banding patterns generated from fixed (F) and unfixed (U) rat pituitary sections are shown. These banding patterns are identical. Additionally, the banding pattern generated from rat pituitary RNA in solution (S) is shown. While there are bands that appear in lane S they are distinct from those that appear in lanes F and U. Arrow indicates the top of the gel. Numbers on the right-hand side indicate the range of size, in nucleotides, of the cDNA bands.

30 ng of primer/100 μ l of hybridization solution) is needed to cover the coverslips. If the primer is labeled with a single ^{32}P moiety using the kinase reaction and no radioactivity is incorporated into the IST cDNA, then the observed intensity of the bands is reflective of the abundance of the band after the IST reaction. The kinase reaction is performed in a 10- μ l final volume with 100 ng of primer, 300 μCi of [γ - ^{32}P]ATP, 1 μ l of $10 \times$ T4 kinase buffer, and 0.5 μ l of T4 polynucleotide kinase. The reaction is

incubated at 37° for 60 min followed by phenol–chloroform extraction and three ethanol precipitations. These reaction conditions usually result in specific activities of between 8×10^8 and 2×10^9 . After hybridization, the sections are washed for 1 hr in $2 \times$ SSC followed by 5 hr in $0.5 \times$ SSC at room temperature. The sections are then washed for 10 min in IST reaction buffer to exchange the SSC salts with IST buffer salts. The IST reaction is performed with dNTPs at a concentration of $250 \mu M$ for 90 min at 37°. The sections are washed briefly (15 min in $0.5 \times$ SSC) and then air dried. X-Ray film autoradiography can be performed to confirm that hybridization and IST occurred. An additional advantage of doing film autoradiography is that the anatomical localization of the IST reaction can be confirmed and may help in directing the removal of the IST derived cDNAs. The IST-derived cDNA transcripts can be removed as described previously. The cDNA is phenol–chloroform extracted and ethanol precipitated. For electrophoretic analysis the cDNA from three coverslips is resuspended in $4 \mu l$ of 3% (w/v) bromphenol blue, 3% (w/v) xylene cyanol, 5 mM ethylenediaminetetraacetic acid (EDTA), and 95% formamide. The cDNA is then electrophoresed on a 6% (w/v) acrylamide gel containing 7 M urea (data not shown).

Interestingly, the autoradiographic intensities of the bands in the banding pattern can be altered by pharmacological manipulation. The intensity of the bands corresponding to larger DNA fragments is increased relative to that of bands corresponding to smaller fragments when the cells were treated with NaF. The relative intensities of the bands are distinct from those generated from untreated cells. Because the specific activity of each band is the same (each band is labeled once at the 5' end of the oligonucleotide primer), the presence of differing intensities for the cDNA bands indicates that there are different molar amounts of cDNAs at each of these bands. NaF, among other things, is reported to inhibit ribosome binding to mRNA (initiation). We tested the ability of NaF to alter the distribution of mRNA in polysomes versus monosomes using sucrose gradient purification of polysomes and found that NaF decreases the number of POMC mRNAs in the polysome fractions of the gradient. This result confirms the hypothesis that a redistribution of mRNA from the highly translated polysome fraction to the low translational monosome fraction is induced by NaF. The parallel to the IST banding pattern is significant, in that if translation of POMC mRNA is decreased, fewer ribosomes would be expected on the mRNA. One interpretation of this result is that the shift in intensity of the IST banding pattern may result from the inability of the reverse transcriptase, which is moving along the mRNA molecule, to progress further down the mRNA before encountering any steric hindrance resulting from binding of multiple ribosomes to the mRNA mole-

cule. The converse of this argument would be that with more ribosomes on a mRNA, i.e., more translation, the ratio of the intensities of the high molecular weight bands to the intensities of the lower molecular weight bands would be decreased.

This hypothesis was tested by adding known modulators of POMC protein production to AtT-20 cells. When 8-bromo-cAMP was given to the cells for 1 hr to stimulate protein synthesis, the ratio of high molecular weight to low molecular weight IST bands was low, while 1 hr of dexamethasone treatment, which decreases POMC protein levels, resulted in reversal of the ratio of the banding pattern. The time course of these treatments was such that no measurable changes in POMC mRNA levels could be detected. These results represent acute responses of the expression of POMC to modulators of POMC production.

Additionally, the differing intensities of the cDNA bands is probably reflecting characteristics of the structure of the mRNA within the cell. With further analysis of the banding pattern and changes that are produced by various modulators, we hope to be able to rigorously correlate IST banding patterns with the cell biology of mRNA interaction with cellular constituents as well as with its translational state.

In Situ Transcription in Single Live Cells

Dissociated Neuronal Cells

The diversity and complexity of mRNAs found in either tissue sections or cells in culture have provided many initial insights into the regulation of gene expression at the transcriptional level. However, one difficult task in molecular biology has been elucidating the diversity and complexity of gene expression within a single cell. Analysis of the total of gene expression in single cells requires that the low level of endogenous mRNA be "amplified" to easily manipulated amounts. Current technology such as the polymerase chain reaction (PCR), while providing a means for amplifying the endogenous signal, does have significant drawbacks when applied to populations of RNAs in single cells. These drawbacks consist of base pair copying errors occurring approximately once every 500 bases and the inability of the enzyme *Taq* DNA polymerase to amplify different lengths of cDNA transcripts linearly with the same efficiency. As a result of this latter observation, the amplified cDNA population is often biased to a population of shorter cDNA transcripts. With these technical obstacles in PCR technology, the use of *in situ* transcription and amplified antisense RNA technologies is more suited to amplifying the mRNA population from tissue sections (as previously described) or single cells.

In situ transcription can be performed in single live neurons.¹¹ The biggest obstacle to performing this reaction initially was the delivery of the IST reagents into the single cell. This hurdle was overcome by adopting the tools of electrophysiology. Using a whole-cell patch electrode, IST of the cellular RNA within a live cell can be accomplished by perfusing the oligo(dT)-T7 primer, dNTPs, and reverse transcriptase from the patch electrode into the cell. The concentrations of these varied components are as follow: 1 μ l oligo(dT)-T7 primer (100 ng/ μ l) [24-nucleotide (nt) poly(T) sequence and 33-nt sequence containing the T7 RNA promoter), 1 μ l each of dNTPs (10 mM) (dATP, dCTP, dGTP, and TTP), 2 μ l 10 $\times$ amplification buffer (1 $\times$ = 154 mM KCl, 6 mM NaCl, 5 mM MgCl₂, 0.1 mM CaCl₂, 10 mM HEPES, pH 8.3), 12 μ l distilled H₂O, and 1 μ l reverse transcriptase (20 units/ μ l).

The perfusion takes approximately 10 min as determined by monitoring the diffusion of fluorescently tagged molecules from the patch electrode throughout the cell. The viability of the cell is also monitored by measuring the resting membrane potential of the cell using the whole-cell patch recording configuration with the IST buffer in place of a standard recording solution. The most variable aspect of this procedure is in the cellular response to the osmolality of the amplification solution. If cell viability is compromised, the osmolality of the solution as determined by the concentration of primer, dNTPs, and amplification buffer can be reduced at least fivefold below that listed by dilution of the amplification mix. Following perfusion, the contents of the single cell are aspirated into the pipette and incubated at 37° for 60 min. Following incubation, the pipette contents are ejected from the pipette and diluted into 50 μ l of TE (10 mM Tris, pH 8.0, 1 mM EDTA). One microgram of tRNA is added as carrier, and the sample is phenol–chloroform extracted followed by ethanol precipitation of the aqueous phase. The cDNA is pelleted, air dried, and resuspended in 20 μ l of distilled H₂O.

Second-strand synthesis is accomplished using DNA hairpinning to produce a primer for DNA synthesis as described in *In Situ* Transcription (above). Specifically, 10 μ l of the first-strand DNA–RNA hybrid, 25 μ l of 2 $\times$ second-strand buffer [800 mM Tris, pH 7.6, 1.0 M KCl, 50 mM MgCl₂, 100 mM (NH₄)₂SO₄, 50.0 μ g/ml bovine serum albumin (BSA)], 2 μ l each of 10 mM dNTPs (dATP, dCTP, dGTP, and TTP), 5 μ l distilled H₂O, and 1 μ l each of Klenow and T4 DNA polymerase are combined. This reaction is performed at 37° for 1 hr. As with the first-strand reaction, the newly synthesized double-stranded cDNA is phenol–chloroform extracted, etha-

¹¹ R. N. VanGelder, M. E. vonZastrow, A. Yool, W. C. Dement, J. D. Barchas, and J. H. Eberwine, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1663 (1990).

nol precipitated twice, and again resuspended in 20 μ l of distilled water after being air dried.

The DNA is next treated with S1 nuclease for 5 min at 37° to eliminate the hairpin formed in the second-strand synthesis reaction. The S1 nuclease reaction is performed by mixing 10 μ l second-strand DNA, 2 μ l 10 $\times$ S1 nuclease buffer (1 $\times$ = 30 mM sodium acetate, pH 4.6, 50 mM NaCl, 1 mM zinc acetate, 7 μ l distilled H₂O, and 1 μ l S1 nuclease (1 unit/ μ l). After S1 nuclease treatment the DNA is phenol–chloroform extracted, ethanol precipitated, and resuspended in 10 μ l of distilled H₂O after air drying.

The next step in the procedure is for the cDNA to be drop dialyzed on a Millipore membrane (0.025- μ m pore size) against distilled H₂O for 4 hr. This step removes the free deoxynucleotide triphosphates remaining from the prior first- and second-strand reactions. If time is of the essence, this step can be accomplished by instead performing two ethanol precipitations of the cDNA. With each ethanol precipitation, approximately 90% of the free triphosphates will be washed away. Of course, with each ethanol precipitation a small amount of cDNA will also be lost. With this in mind, because the quantity of starting material is only between 0.1 and 1.0 pg, any loss of the dsDNA may amount to losing a significant proportion of the population and may result in a less accurate representation of the original population. For this reason, the preferred method of free triphosphate elimination is the use of drop dialysis.

With the completion of this step, the dsDNA has been constructed to contain the T7 RNA polymerase promoter such that antisense RNA will be made when T7 RNA polymerase is added to initiate RNA synthesis. While it is convenient to use the Promega Riboprobe Gemini System II buffer kit, all the solutions and buffers can be made up independently. It is crucial that all solutions and buffers be RNase free. RNA amplification is performed by adding the following reagents: 4 μ l 5 $\times$ buffer (200 mM Tris-HCl, pH 7.9, 30 mM MgCl₂, 10 mM spermidine, 50 mM NaCl), 4 μ l of double-stranded cDNA to be amplified, 1 μ l of 100 mM DTT, 1 μ l each of 10 mM stocks of NTPs, 1.5 μ l distilled H₂O, 2 μ l [³²P]CTP (10 mCi/ μ l), 0.5 μ l RNasin (50 units/ μ l) and 1 μ l T7 RNA polymerase (2000 units/ μ l). The aRNA amplification is carried out at 37° for 2–4 hr. To determine molar amplification, 0.5 μ l of the reaction mix is spotted on 1-mm Whatman paper immediately after the addition of the T7 RNA polymerase and a second time after the reaction is complete. The TCA (CCl₃COOH)-precipitable radioactivity will not only give indications of how well the reaction worked but also the molar amplification. After the reaction is finished, the product is phenol–chloroform extracted and ethanol precipitated. The pellet is resuspended in 20 μ l of distilled H₂O and is ready for use.

While the molar amplification of the cDNA often varies due to the reaction conditions, up to 2000-fold amplification can be routinely achieved. The aRNA population synthesized using this procedure is proportional in abundance to the initial mRNA population. This has been determined by comparing the relative abundance of several specific mRNAs by single-cell expression profiling with the abundance of multiple cells using the standard Northern blotting procedure.

First, the amplified RNA population is now antisense to the original mRNA population.¹¹ In such form, it can be used directly as a probe either for screening blots or libraries. Second, the population of mRNAs is present in the same proportions as in the original population. There appears to be little skewing of the amplification process. Additionally, the aRNA population size range is the same as the cDNA and not biased toward shorter sequences as is true for the PCR.

In spite of molar amplification up to 2000-fold, the amplification of the mRNA from the single cell still represents between only 0.2 and 2 ng of RNA. While this limited amount of material may be adequate for screening blots and libraries, a second round of amplification is often necessary to construct libraries from single cells and to examine low-abundance messages that may not exhibit significant hybridization with the 2000-fold amplified RNA. For this purpose a second round of cDNA synthesis is performed (see Amplified Antisense RNA Amplification, above). The aRNA resulting from two rounds of amplification of the original material from a single cell results in sufficient material to perform expression profiling, screening, and construction of cDNA libraries.

Single Cells within Thick Tissue Slices

The study of gene expression in individual cells may also be performed in slice preparations. The major advantage of this approach is that the cell under investigation remains in an immediate environment that has not been experimentally perturbed. This means, in studies of the nervous system, that neurons maintain many of their normal synaptic connections while the experiment is being performed. The application of patch clamp technology to tissue sections of varying thickness¹² makes this approach feasible. Preliminary experiments in our laboratory have included studies of the hippocampus isolated from newborn rats.

After decapitation and gross dissection of the brain on ice (rat pups ranging in age from 5 to 28 days), sections of 100 to 200 μm in thickness were cut on a Vibratome set at maximum vibration. Several slices are

¹² F. A. Edwards, A. Konnerth, B. Sakmann, and T. Takahashi, *Pfluegers Arch.* **414**, 600 (1989).

preincubated at 37° in physiological saline solution (125 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 25 mM glucose, 26 mM NaHCO₃, and 1.25 mM NaH₂PO₄) for 1–4 hr. An individual slice is mounted onto a modified microscope stage that includes a water immersion lens and differential phase-contrast optics. A broken micropipette is positioned directly above pyramidal cells (any cells that are recognized by morphological criteria may be chosen). The superficial surface above the cell is partially removed via gentle suction and blowing. This cleaning step ensures the development of a tight seal with the patch clamp electrode. An RNase-free microelectrode is next used in the formation of a whole-cell clamp. The interior of the microelectrode contains the necessary reagents for first-hand cDNA synthesis. The contents of this microelectrode are processed in a manner identical to that described previously for dissociated cells in culture (see Dissociated Neuronal Cells, above). The average size of amplified aRNA obtained from a hippocampal pyramidal neuron in the CA1 region in this experiment was 800 bases, with the highest size visible by autoradiography being approximately 3 kb. In expression profiling this aRNA hybridized to cDNAs for the muscarinic and γ -aminobutyric acid (GABA)-A receptors, which were immobilized on nitrocellulose paper.

Summary

In situ transcription is the synthesis of cDNA within cells. This chapter has illustrated some of the applications of IST to the study of gene expression in complex cell environments. While the importance of transcription in modulating cellular activity has been long appreciated, the role of translational control mechanisms in regulating central nervous system functioning is just beginning to be recognized. Previous limitations in the availability of tissue have made it difficult to construct cDNA libraries from defined cell populations, to examine translational control, and to quantitate differences in the amount of mRNA for many distinct mRNAs in the same sample. *In situ* transcription facilitates all of these procedures, making it possible to characterize aspects of gene regulation that were previously difficult. Indeed, taken to its furthest extreme it is now possible to characterize gene expression in single live cells. This level of analysis allows basic questions, such as How different morphologically identical cells are at the level of gene expression, and How synaptic connectivity and glial interactions influence gene expression in single cells, to be experimentally approached. The ability to characterize gene expression in small amounts of tissue and single cells is critical to gaining an understanding of the contribution of specific cell types to the physiology of the central nervous system.

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[10] Specific Amplification of Complementary DNA from Targeted Members of Multigene Families

By Z. CAI, J. K. PULLEN, R. M. HORTON, and L. R. PEASE

Introduction

The polymerase chain reaction (PCR) provides convenient strategies using cDNA to study the structural and functional properties of proteins. However, the coexistence of transcripts in many cell types from structurally related members of multigene families complicates the identification and analysis of specific mRNA species. Polymerase chain reaction amplification artifacts have been reported when more than one homologous transcript serves as the template for the PCR primers.¹⁻³ Typical among these artifacts are recombinants in which sequences from transcripts derived from different loci have been scrambled. The frequency of such recombination has been reported as 8 and 5%.^{1,2} Excision repair of heteroduplexes during cloning could lead to higher frequency of recombination (25%) in cloned PCR fragments.³

Two strategies have been employed to overcome this problem.^{1,4} The first is to sequence many clones from independent amplification reactions to establish the consensus sequence of each cDNA. Typically, nonrecombinant clones represent the majority of sequences identified and specific recombinant motifs tend to be unique, although secondary structures in the amplified templates can lead to the independent generation of identical mutants. The second approach has been to identify sequences in the mRNA, preferably in the 5' and 3' untranslated regions of the transcript, that provide locus specificity. This approach can yield highly specific

¹ P. D. Ennis, J. Zemmour, R. D. Salter, and P. Parham, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2833 (1990).

² A. Meyerhans, J.-P. Vartanian, and S. Wain-Hobson, *Nucleic Acids Res.* **18**, 1687 (1990).

³ R. Jansen and F. D. Ledley, *Nucleic Acids Res.* **18**, 5153 (1990).

⁴ Z. Cai and L. R. Pease, *Immunogenetics* **32**, 456 (1990)