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# Isolation of enzyme cDNA clones by enzyme immunodetection assay: Isolation of a peptide acetyltransferase

(λgt11 screening/enzyme activity assay/acetylated peptides)

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ABSTRACT **The biological activity of many proteins and peptides can be profoundly affected by enzyme-catalyzed covalent modifications such as acetylation, sulfation, glycosylation, or amidation. This article describes the cloning of such an enzyme, a peptide acetyltransferase from rat brain that catalyzes the amino-terminal acetylation of endorphins and perhaps other substrates *in vivo*. Blot-hybridization analysis suggests that the mRNA encoding the acetyltransferase is ≈2.0 kilobases, is present in whole rat brain and rat hypothalamus, and is slightly larger in mouse AtT20 tumor cells. The acetyltransferase was cloned by using a strategy whereby a cDNA expression library was screened with a solid-phase enzyme-activity assay; this technique combines the use of the substrate coupled to a solid support and subsequent recognition of the product by using a specific antiserum. We have called this method the enzyme immunodetection assay (EIDA). The EIDA should prove useful in the isolation of other clones for proteins that possess enzymatic activity upon expression in bacterial hosts.**

Amino-terminal acetylation is a physiologically important posttranslational modification that can modulate the bioactivity of a number of molecules. Both α-melanotropin (α-MSH) and β-endorphin, which are derived from the same precursor protein, proopiomelanocortin (1), are acetylated at the amino terminus in melanotrophs of the rat intermediate pituitary gland (2–4). However, the β-endorphin in human adrenal anterior-lobe corticotrophs and in brain is predominantly the nonacetylated species (5–7). Whereas unmodified β-endorphin is a potent opioid peptide, the acetylated species does not bind to opioid receptors and, consequently, cannot elicit a physiological response (8, 9). In contrast, acetylation can enhance biological activity, as evidenced by α-MSH, which is a considerably more potent melanocyte-stimulating hormone *in vivo* than is the nonacetylated derivative (10).

Aside from a potential role in the regulation of the activity of peptide hormones, acetylation is also a common modification of histone proteins (11). The degree of histone acetylation has been shown to alter the ability of some histones to bind to DNA, leading to speculation that acetylation plays a role in the regulation of gene transcription (12).

Acetylation is clearly an important covalent modification of peptides and proteins, yet little is known about the cellular localization or regulation of the enzyme(s) catalyzing amino-terminal acetylation. An understanding of the enzyme would be greatly enhanced by the availability of antibodies and cDNA clones, allowing the measurement of protein and mRNA levels. With these tools, the important question of whether coregulation occurs between a substrate (proopiomelanocortin) and one of its processing enzymes can be addressed. In view of these goals, the cloning of cDNA encoding an acetyltransferase activity was initiated.

## MATERIALS AND METHODS

**Preparation of Substrate Paper.** Aminophenyl thioether paper (APT) (13) was cut to fit 150-mm diameter Petri dishes. The cut paper was activated by incubation in 0.5 liter of 1.2 M HCl containing 135 mg of $NaNO_2$ for 20 min at 0–4°C, at which stage the paper turned a bright yellow color. The paper was washed three times in ice-cold water and then once in ice-cold 15 mM phosphate buffer (pH 6.5). The activated paper was placed in the Petri dish containing 1 mg of the *t*-butoxycarbonyl (Boc) derivative of [Leu$^5$]enkephalin hydrazide (Biosearch, San Rafael, CA) dissolved in 50 ml of 15 mM phosphate buffer, pH 6.5/50 mM NaCl/5% (wt/vol) acetonitrile. The peptide was incubated with the activated paper for 24 hr at 4°C, after which the complex was placed in 100 ml of 100 mM Tris·HCl, pH 9.0/2.5% (wt/vol) gelatin for 2 hr at room temperature to block remaining azide groups. At this step the paper turned a brick-red color. Finally, to remove the Boc group from the coupled peptide, the paper was immersed in 1.2 M HCl for 30 min at room temperature. The paper was washed in distilled water and stored at −20°C until required.

**Screening of Phage Libraries.** The library was plated as described (14). After 6 hr of incubation at 39°C, substrate-coupled paper previously soaked in 25 ml of cofactor solution (25 mM Tris·HCl, pH 7.0/0.25 M sucrose/50 mM KCl/4 mM ATP/4 mM $MgCl_2$ (15–17)/5 mM acetyl-CoA) was placed on top of the bacteriophage lawn. This was incubated for as short a time as 30 min or as long as overnight with 300 μl of fresh cofactor solution dropped on and spread over the filter every hour. The product–paper complex was then removed, washed for 3 hr with 1× NET buffer (120 mM NaCl/1 mM EDTA/20 mM Tris·HCl, pH 7.5) and incubated with antibodies to *N*-acetyl-β-endorphin overnight at 4°C with gentle shaking. The excess antibody was removed by washing with several changes of 1× NET buffer at 4°C. The bound antibodies were detected by first incubating the filters with $2 \times 10^6$ dpm of $^{125}$I-labeled protein A overnight at 4°C. The unbound protein A was removed by washing with several changes of 1× NET, and the filter was air-dried and autoradiographed at −70°C for 2 days with a Cronex intensifying screen.

**HPLC of the Supernatant from Liquid-Phase EIDA.** Bacteria were infected with positive or negative phage and incubated at 39°C for 4 hr to induce lysis. Isopropyl thiogalactoside was added to a concentration of 10 mM and incubated for 30 min. This was followed by addition of [Leu$^5$]enkephalin to a final concentration of 1 mM, and the

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Abbreviations: EIDA, enzyme immunodetection assay; MSH, melanotropin (melanocyte-stimulating hormone); Boc, *t*-butoxycarbonyl; APT, aminophenyl thioether.

medium was adjusted so that it contained all of the components of the cofactor solution (see Fig. 1). After 4 hr at 39°C, the samples were centrifuged at 10,000 × *g* for 10 min, and the supernatant was frozen to stop enzyme activity. Samples were then chromatographed on a 5-μm 250 mm × 4.6 mm $C_{18}$ HPLC column (Altex ultrasphere ODS) with a buffer containing 50 mM monosodium phosphate and 5% methanol, brought to pH 2.7 with phosphoric acid. The peptides were eluted with an acetonitrile gradient as shown in Fig. 1. Fractions of 1.25 ml were collected every minute. Aliquots were evaporated to dryness and then assayed with the acetyl-specific RIA.

**RNA Isolation and Blot-Hybridization Procedures.** Total RNA was isolated from tissues of interest by using the method of Chirgwin *et al.* (18). The RNA was quantitated by measuring absorbance at 260 nm. RNAs were stored in ethanol until used. Total RNA (30 μg) was heat-denatured at 90°C for 3 min, quick-cooled on ice, and electrophoresed on 1.5% agarose/formamide/formaldehyde gel (19). The RNA was transferred to nitrocellulose (20), and the filter was baked under vacuum at 90°C for 4 hr. Then the filter was prehybridized and hybridized according to Wahl *et al.* (14). The probe utilized in the hybridization was nick-translated (21) ($4 \times 10^8$ cpm/μg) A7 DNA, a 2-kilobase (kb) *Eco*RI fragment of the parent EEB7 phage, which had been subcloned into the pUC18 vector.

**DNA Isolation and cDNA Subcloning.** Bacteriophage DNA was isolated from plate lysates by using the procedure of

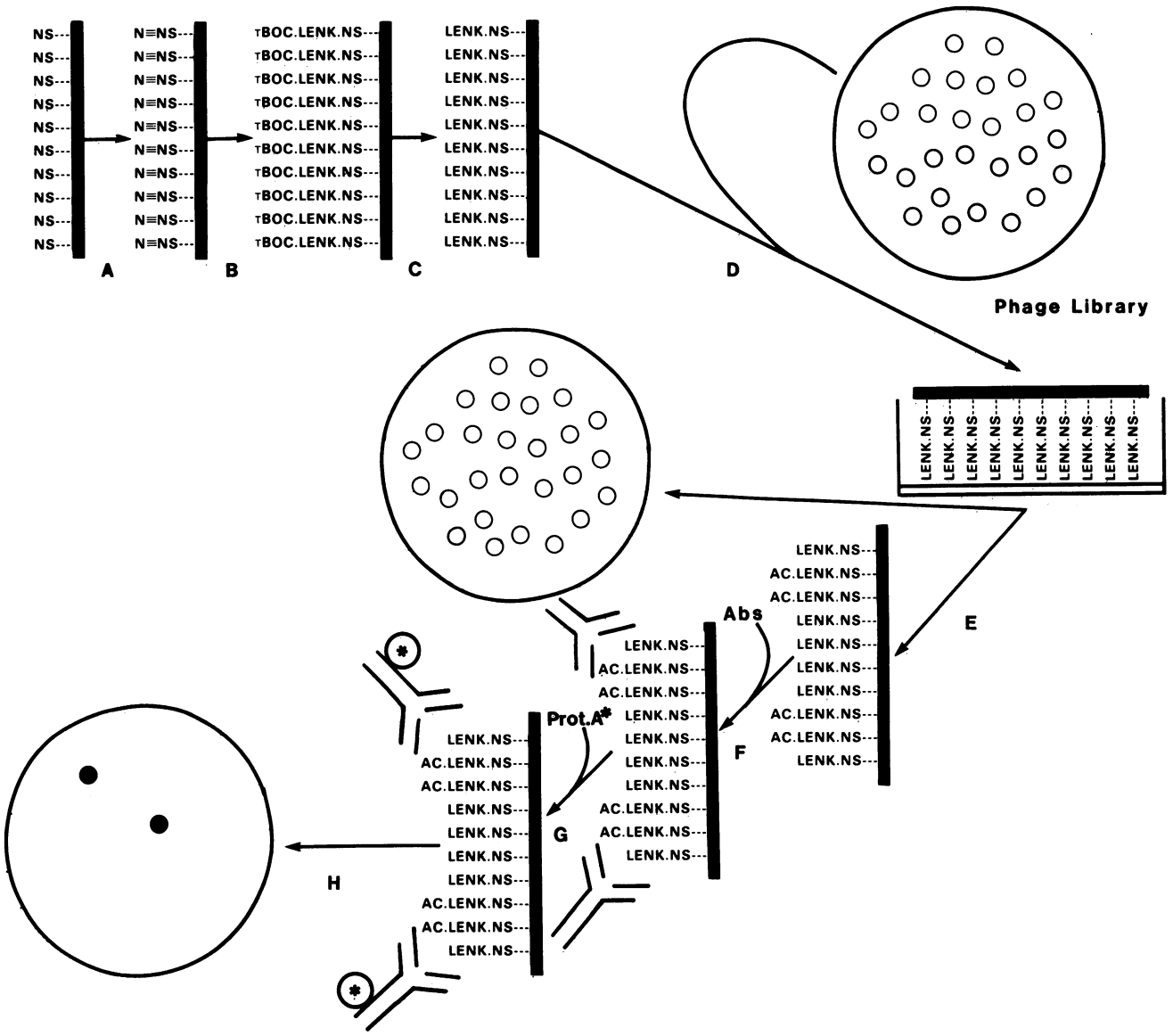


FIG. 1. Schematic of the EIDA assay used for cloning the acetyltransferase. For coupling of the peptide to a solid support, APT paper was chosen, which consists of cellulose linked covalently to a spacer arm (S) to which an aromatic amine is attached (N). This aromatic amine can be readily converted to the azide (step A) by treatment with nitrous acid. The resulting azide reacts readily with nucleophiles such as primary amines. In order to covalently couple [Leu$^5$]enkephalin to the paper via the carboxyl terminus (step B), a modified enkephalin was used (tBOC.LENK) that was protected at the amino terminus by a Boc group and derivatized at the carboxyl terminus to form the carboxyl-terminal hydrazide, providing a nucleophilic center. After coupling of the peptide, the remaining azide groups on the paper were deactivated, and the Boc group was removed by acid treatment revealing the α-amino group required for enzymic addition of the *N*-acetyl group (step C). This step completed the synthesis of the substrate paper. The λgt11 cDNA library representing mRNA from whole rat brain was plated out in Petri dishes. Substrate paper soaked in the enzyme assay buffer was laid on top of the library immediately following induction of bacterial lysis. After 4 hr at 37°C, the paper was removed, and the Petri dish containing the library was stored at 4°C. Areas of the paper converted to the α-N-acetylated product were recognized by acetyl-specific antisera (Abs), which were incubated with the paper for 24 hr at 4°C (step F). Bound antisera were visualized by $^{125}$I-labeled protein A (Prot.A*), followed by autoradiography (steps G and H). By matching dark areas on the autoradiogram with plaques, clones producing acetyltransferase-like activity could be identified.

Yamamoto *et al.* (22). The phage DNA was then digested with the restriction endonuclease *Eco*RI, and the liberated cDNA insert was subcloned into the *Eco*RI site of the pUC18 plasmid vector. This plasmid DNA was then transformed into DH1 (37) bacteria by $CaCl_2$ precipitation (23). The resultant colonies were screened for the presence of the cDNA insert (by cDNA size analysis) and then grown in large quantity. Plasmid DNA was isolated by the method of Ish-Horowicz and Burke (24). The resultant DNA was used for probe preparation.

## RESULTS

The EIDA technique requires (*i*) the availability of a peptide or protein substrate that can be covalently bound to paper, (*ii*) a cDNA expression library containing full-length or near-full-length cDNAs made from mRNA isolated from a tissue expressing the enzyme of interest, and (*iii*) antibodies that will discriminate between the enzyme substrate and the product. APT paper (25) was chosen as the solid-phase substrate support because it provides a nonreversible covalent attachment of peptides via their amino groups in a diazotization reaction. The modified enkephalin was bound to activated ATP paper via the carboxyl-terminal hydrazide so that the peptide binding capacity, estimated from previous tracer studies, was $\approx 3\ \mu g/cm^2$. After the modified enkephalin was coupled to the paper, the protecting Boc group was removed by acid treatment, exposing the free $\alpha$-amino group of the amino-terminal tyrosine to which the acetyl group could be attached by an *N*-acetyltransferase.

The cDNA library used in this study was made from rat brain poly(A)$^+$ RNA by using G-tailing and oligo(dC)-priming to synthesize the second cDNA strand provided by V. Alberts, E. Beatge, and T. Joh, Department of Neurobiology, Cornell Medical Center, New York, NY). This method favored cloning of full-length double-stranded cDNAs necessary for this type of assay. The cloning vector $\lambda$GT11 is a bacteriophage $\lambda$ expression system that generates a fusion protein consisting of $\beta$-galactosidase and the amino acid sequence specified by the cloned insert cDNA (26).

Detection of the acetylated product was achieved by using an antiserum generated in rabbits that is specific for $\alpha$-*N*-acetyl-Tyr-Gly-Gly-Phe-Xaa (27), in which Xaa is an unknown amino acid. The antiserum requires the acetyl group as part of the antigenic site and, consequently, will distinguish between the substrate and the N-acetylated product. The mechanics of the EIDA procedure are detailed in the legend to Fig. 1.

Screening of 60,000 clones revealed 1 positive clone (Fig. 2 *Upper*). The positive clone was replated at low density and subjected to the identical screening procedure. Two autoradiograms showing the second-round positives plated at different times with two different substrate–paper complexes are shown in Fig. 2 *Lower*. The presence of approximately the same number of second-round positives on papers from two different platings ($\approx$100 phage per plate) indicates that the positives are specific to a subset of the infecting phage. The acetylating-enzyme phage clones were plaque-purified to homogeneity by a third round of screening, and a restriction endonuclease map is presented in Fig. 3.

Potential problems with this screening procedure are the high concentration of primary antiserum used to identify the product and/or the $^{125}$I-labeled protein A used to identify bound antiserum, both of which could permit the detection of false positives. To address this concern, we performed liquid-phase EIDA; this involved growth of the acetylating-enzyme phage in liquid culture for 4 hr, addition of buffer and substrate, and detection of the presence of acetylated $\beta$-endorphin in the cultures by using a competitive RIA directed to $\alpha$-N-acetylated endorphins. Typically, the antiserum used

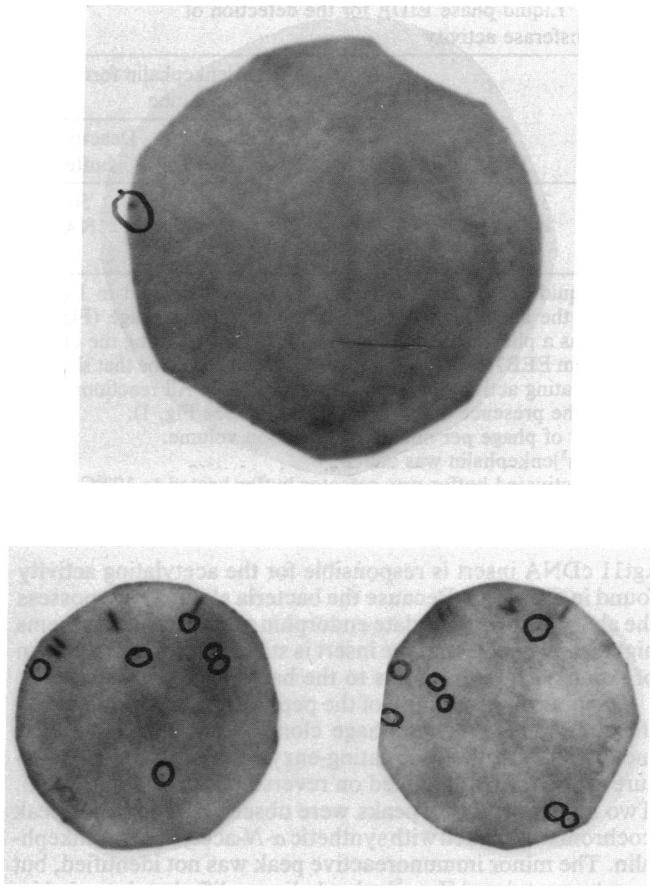

FIG. 2. EIDA-filter screening positives. Primary and secondary screens with the EIDA assay of the rat brain $\lambda$gt11 cDNA library for a peptide acetyltransferase. (*Upper*) The primary screen positive is indicated by a circle. (*Lower*) Secondary positives are circled.

in this RIA is diluted 1:50,000, thus increasing the specificity manyfold over the 1:600 dilution used in the solid-phase EIDA assay. The RIA results of this assay are presented in Table 1. Clearly, at a given phage titer, the acetyltransferase is capable of producing a large quantity of acetylated immunoreactive product from the endorphin substrate.

No enzyme activity could be detected when both the ATP and acetyl-CoA were hydrolyzed by heating prior to assay (Table 1). Additionally, when a randomly picked negative clone was analyzed, no acetyltransferase activity was observed, demonstrating the specificity of the assay. As a further control, the cDNA insert of the positive clone was reversed in the phage vector, and the activity was rechecked by the liquid-phase EIDA. This construction resulted in very little acetyltransferase activity. These results show that the

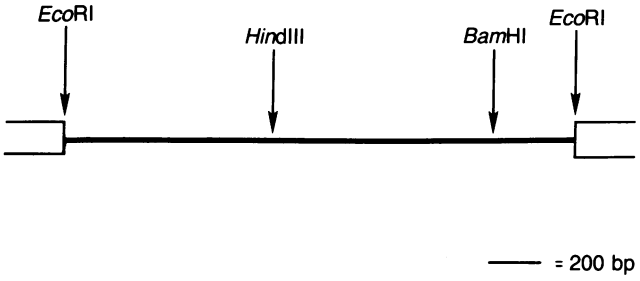


FIG. 3. Restriction map of acetyltransferase clone. The cDNA encoding the acetyltransferase is $\approx$2 kb in length. The positions of *Bam*HI and *Hin*dIII restriction enzyme cleavage sites are as indicated. bp, Base pairs.

Table 1. Liquid-phase EIDA for the detection of acetyltransferase activity

| Clone | Titer* | *N*-Acetyl-[Leu$^5$]enkephalin formed, fmol per tube | | |
|---|---|---|---|---|
| | | EIDA | Without substrate† | Deactivated buffer‡ |
| EEB7 | $3.0 \times 10^{10}$ | 466 | 42 | 50 |
| BEE7 | $4.6 \times 10^{10}$ | 18 | 21 | NA |
| RPC1 | | 36 | | |

The liquid-phase EIDA was performed as described in Fig. 2. EEB7 is the active acetylating enzyme-containing phage (Fig. 1); BEE7 was a phage construction resulting from inverting the cDNA insert from EEB7; and RPC1 is a randomly picked clone that showed no acetylating activity in the solid-phase EIDA. All reactions were done in the presence of 1× cofactor buffer (see Fig. 1).
*Number of phage per milliliter of reaction volume.
†No [Leu$^5$]enkephalin was added.
‡The deactivated buffer was cofactor buffer heated to 100°C for 10 min. NA, not assayed.

λgt11 cDNA insert is responsible for the acetylating activity found in the clone. Because the bacteria alone do not possess the ability to α-N-acetylate endorphin or enkephalin, it seems highly improbable that the insert is stimulating the expression of an enzyme endogenous to the bacterial host.

To confirm the identity of the peptide product produced by the acetylating-enzyme phage clone, [Leu$^5$]enkephalin was incubated with the acetylating-enzyme phage, and the mixture was chromatographed on reverse-phase HPLC (Fig. 4). Two immunoreactive peaks were observed. The major peak cochromatographed with synthetic α-*N*-acetyl-[Leu$^5$]enkephalin. The minor immunoreactive peak was not identified, but may be acetylated [Leu$^5$]enkephalin modified or degraded by other bacterial enzymes.

Blot-hybridization analysis (Fig. 5) of RNA extracted from rat whole brain, hypothalamus, and mouse AtT20 cells showed that the size of the acetyltransferase mRNA in those tissues was about 2.0 kb. Molecular size of the hybridizing band in the mouse AtT20 tumor cell RNA was slightly larger than those from rat brain and hypothalamus.

## DISCUSSION

The data presented demonstrate that the cloned enzyme can α-N-acetylate β-endorphin (a natural substrate for peptide acetyltransferase in the pituitary), [Leu$^5$]enkephalin, and [Leu$^5$]enkephalin hydrazide bound to APT paper. Although considerable enzyme activity capable of α-N-acetylating β-endorphin has been described in brain tissue *in vitro*, little or no α-N-acetylated enkephalin (6), α-N-acetylated β-endorphin (6), or α-MSH can be found in brain (28). Furthermore, the specific localization of acetylating activity that others have described does not mirror the distribution of the opioid peptides, suggesting that these endogenous opioid peptides may not be the natural substrates for the brain acetylating enzyme (29). Since this enzyme clone was isolated from a cDNA library derived from rat brain, it will be particularly relevant to analyze the substrate specificity of the recombinant and natural enzyme (15) with regard to histones (11), serotonin (30, 31), and α-MSH, which has been shown to be O-acetylated as well as α-N-acetylated in some tissues (28, 32). Additionally, the difference in rate of acetylation of isoniazid and other drugs is probably due to differences in the amount or type of acetyltransferase (33). Again it will be important to determine whether the enzyme clone will function on drugs such as isoniazid (33), aniline (34), and sulfonamides (35). The question of specificity is particularly important because blot-hybridization analysis indicates that the acetyltransferase mRNA is in high abundance, since it was detected in whole-brain RNAs. The size of the RNA, about 2.0 kb, indicates that it has enough coding capacity to code for a 47-kDa protein, which is the size of a pituitary peptide acetyltransferase as determined by target analysis (36). Whether the difference in size of AtT20 acetyltransferase mRNA from that of rat brain is due to species or tissue differences is not known.

With respect to the tissue distribution of acetyltransferase mRNA, it is important to note that the presence of mRNA

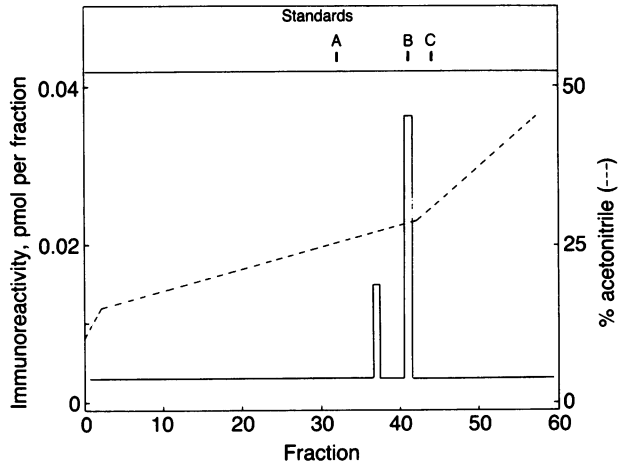


FIG. 4. HPLC analysis of the enzyme reaction product. Reverse-phase HPLC characterization of the immunoreactive material detected by liquid-phase EIDA after incubation of [Leu$^5$]enkephalin with positive-phage culture. Standards: A, [Leu$^5$]enkephalin; B, α-*N*-acetyl-[Leu$^5$]enkephalin; C, $^{125}$I-labeled [Leu$^5$]enkephalin. No immunoreactive material was detected with the negative phage.

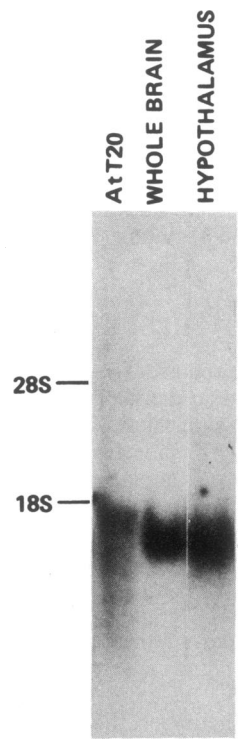


FIG. 5. Blot-hybridization analysis of acetyltransferase mRNA. Thirty micrograms of total RNA isolated from AtT20 cells, rat whole brain, and rat hypothalamus was electrophoresed on a 1.5% agarose/formamide/formaldehyde gel. The position of the residual 18S and 28S ribosomal RNA bands was visualized by ethidium bromide staining after transfer of the RNA to nitrocellulose. Autoradiographic exposure was at −80°C with a Cronex intensifying screen for 11 days.

does not necessitate that the mRNA be translated into functional enzyme. It would appear from the work of O'Donohue and Chappell (29) that an enzyme with similar characteristics may indeed be functional in diverse brain regions.

The general usefulness of the EIDA technique for cloning cDNAs coding for enzymes is dependent on a number of factors. The enzyme of interest must be composed of a single subunit, and its activity must not be entirely dependent on posttranslational modifications that the bacterial host cannot perform, such as glycosylation. The activity must be distinguishable from the enzyme repertoire of the bacteria, and the degradation of the substrate and the product by bacterial enzymes should be minimal. The latter problem may be avoided by the use of a strain of bacteria deficient in the major proteolytic-degrading enzyme (lon mutants) (26). Finally, the assay as described in this instance utilized an antiserum specific for the product that did not recognize the substrate. This may be a difficult tool to generate for many enzyme products, but conceivably other detection methods can be utilized. Although the EIDA screening assay has limitations, the successful isolation of a cDNA clone encoding an enzyme that catalyzes the $\alpha$-N-acetylation of opioid peptides suggests that the technique may have broad applicability. We anticipate that the solid- and liquid-phase EIDA or similar assay procedures based on the principle of screening cDNA expression libraries for enzymatic activity will be useful in leading to the structure of other enzymes that catalyze posttranslational modifications.

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