Comparative Expression of Homologous Proteins

A NOVEL MODE OF TRANSCRIPTIONAL REGULATION BY THE CODING SEQUENCE FOLDING
COMPATIBILITY OF CHIMERAS*

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Recombinant acetylcholinesterases (AChE) are produced at systematically different levels, depending on the enzyme species. To identify the cause of this difference, we designed expression vectors that differed only by the central region of the coding sequence, encoding 
Torpedo, rat, and Bungarus AChEs and two reciprocal rat/Bungarus and Bungarus/rat chimeras. We found that folding is a limiting factor in the case of 
Torpedo AChE and the chimeras, for which only a limited fraction of the synthesized polypeptides becomes active and is secreted. In contrast, the fact that rat AChE is less well produced than 
Bungarus AChE reflects the levels of their respective mRNAs, which seem to be controlled by their transcription rates. A similar difference was observed in the coding and noncoding orientations; it seems to depend on multiple cis-elements. Using CAT constructs, we found that a DNA fragment from the 
Bungarus AChE gene stimulates expression of the reporter protein, whereas a homologous fragment from the rat AChE gene had no influence. This stimulating effect appears different from that of classical enhancers, although its mechanism remains unknown. In any case, the present results demonstrate that the coding region contributes to control the level of gene expression.

The biosynthesis of proteins is highly controlled, and its regulation has been studied extensively. The production of an active protein involves multiple steps, including gene transcription, mRNA processing, translation, and various post-translational processes such as protein folding, modification, assembly, transport, and targeting to its functional destination. Each of these steps can be separately regulated, and may be critical in the context of normal cells, depending on developmental or physiological conditions, or limiting for the production of recombinant proteins in transfected cells.

However, analyses of the regulation of gene activity have mostly focused on the role of promoters and enhancers on transcription. In a frequently used experimental paradigm, the efficiency of a promoter is analyzed in constructs where more or less extended fragments of the 5′ region preceding the transcription site control the expression of a reporter protein, such as β-galactosidase, luciferase, or chloramphenicol acetyltransferase (CAT). This protocol is convenient and allows a systematic exploration of the upstream promoter region, but it neglects the regulatory elements that may be located downstream of the transcription initiation site, especially within the transcribed region, although the existence of such downstream regulatory elements has indeed been reported, particularly in introns. Thus, the possible influence of the coding sequence on transcription is generally ignored. In addition, the properties of the protein itself, such as its folding efficiency, may limit the production of a functional molecule.

On the contrary, the present study focuses on the role of coding sequences and on the intrinsic properties of proteins. It arose from the observation that highly homologous acetylcholinesterases (AChEs) from different species are produced at markedly different levels in transfected cells, in the same expression vectors. In particular, more AChE from the snake 
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* This work was supported by grants from the Centre National de la Recherche Scientifique, the Association Française contre les Myopathies, the Direction des Forces et de la Prospective, and the European Community. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: CAT, chloramphenicol acetyltransferase; AChE, acetylcholinesterase; kb, kilobase(s); PAGE, polyacrylamide gel electrophoresis.

2 N. Morel, unpublished observations.

3 I. Shin, I. Silman, C. Bon, and L. Weiner, personal communication.
Coding and Peptide Sequences Control Protein Expression

MATERIALS AND METHODS

Plasmid Constructions—The structure of our plasmid constructs is schematically shown in Fig. 1. The coding sequences from Torpedo, Bungarus, and rat AChEs were inserted in the same expression vector, pcDNA3 (Invitrogen). The sequences encoding the signal peptides of rat and Torpedo AChEs was generally replaced by the corresponding Bungarus sequence. We use lowercase letters (b, r, and t for Bungarus, rat and Torpedo, respectively) to specify coding sequences corresponding to the signal peptides, and capital letters (B, R, and T) for the mature peptide, in constructs, the native XbaI site was replaced by a corresponding SphI site (B2, R2, and T2). Thus, br and bR2 correspond respectively to rat AChE and to a rat/Bungarus chimera, both with the signal peptide from Bungarus AChE (see Fig. 1). The rat and Bungarus constructs encoded the C-terminal peptide ATEVPC, derived from the first six residues of the rat H peptide. The Torpedo constructs encoded the C-terminal peptide ACPG. The lack of an anchoring signal, such as a glycolipid addition signal, and the presence of a cysteine allowed the production of secreted soluble dimers in all cases.

For construction of pcDNA3/bT expressing dimeric Torpedo AChE with the signal peptide of Bungarus AChE, we introduced a KpnI site at the 5′ end of the coding sequence of Torpedo marmorata AChE, a stop codon, and an XbaI site after the cysteine encoded by the H exon, in pEF/BOS AChE Eff (4). In addition, the only free cysteine within the catalytic domain, C231, was substituted by a serine. The KpnI/XbaI fragment was then inserted into pcDNA3 (Invitrogen) to give pcDNA3/bT. To replace the Torpedo signal peptide by that of Bungarus AChE, a MfeI site was introduced between the regions encoding the signal peptide and the catalytic domain of Torpedo AChE. The region encoding the signal peptide of Bungarus AChE was amplified by polymerase chain reaction, using the T7 primer and a primer corresponding to the end of the signal peptide, including an EcoRI site. The polymerase chain reaction product was then digested by HindIII and EcoRI and ligated to the 7-kb HindIII/MfeI fragment of pcDNA3/Tt, producing pcDNA3/bT.

For construction of pcDNA3/bR, we inserted a sequence encoding the peptide ATEVPC by site-directed mutagenesis, at the end of the region encoding the catalytic domain of Bungarus AChE without a C-terminal peptide, in the pcDNA3/NAT vector (1).

For construction of pcDNA3/bR, a stop codon and an XbaI site were inserted after the cysteine codon of the rat H exon in the pEF-Bos vector expressing rat AChE Eff. After digestion by EcoRI and XbaI, the 1.75-kb fragment containing the coding sequence of rat AChE was inserted into pcDNA3. The rat peptide signal was replaced by that of Bungarus, as described above.

For construction of a rat/Bungarus chimera (br/bR2), a 0.72-kb HindIII/SphI fragment of pcDNA3/bR and a 1.7-kb SphI/XhoI fragment of pcDNA3/bR were inserted between the HindIII/XhoI sites of pcDNA3.

For construction of a Bungarus/rat chimera (br/R2), a 0.72-kb HindIII/SphI fragment of pcDNA3/bR was ligated with a 0.9-kb SphI/BamHI fragment and a BamHI/HindIII 5.7-kb fragment of pcDNA3/hR2.

For the construction of inverted constructs, invBr and invbR, the respective inserts were amplified by polymerase chain reaction and inserted at the EcoRV site of pcDNA3, in the noncoding orientation.

For CAT constructs, we inserted the CAT gene under the cytomegalovirus promoter, upstream of the polyadenylation site of the pcDNA3 vector (CAT-pA construct). To this construct, we added the bR2 fragment (718 bp), digested after the CAT coding sequence, either upstream or downstream of the polyadenylation site (CAT-bR2-pA and CAT-pA-bR2, respectively); CAT-brR2-pA, CAT-rR2-pA, and CAT-pA-R2 were constructed in the same manner.

Transfections and Cell Cultures—COS cells were transfected by the DEAE-dextran method, as reported previously (5), using 6 μg of DNA encoding AChE/10-cm dish. The cells were maintained at 37 °C in a medium containing Nuserum (Inotech, Dottikon, Switzerland). C2 cells were transfected with Fugene (Amersham Pharmacia Biotech) and were maintained 3–4 days in Dulbecco's modified Eagle's medium supplemented with 10% of horse serum and 20% of fetal calf serum (Life Technologies, Inc.). In some experiments, the medium was changed 2 days after transfection, and the transfected cells were incubated with cycloheximide (100 μg/ml) (Sigma) or with actinomycin D (50 μg/ml) (Sigma) for the indicated period of time before RNA extraction.

Expression in Xenopus Oocytes—Xenopus oocytes were injected with 5 ng of mRNA into the cytoplasm or with 50 pg of cDNA into the germinal vesicle and were incubated in Barth medium at 19 °C. Synthetic transcripts were injected with the Ambion mMachine™ in vitro transcription kit (Ambion, Austin, TX) using pcDNA constructs as templates.

AChE Activity Determination—For determination of AChE activity, the cells were extracted 3–4 days after the transfection, in a buffer containing 50 mM Tris-HCl, pH 7, 10 mM MgCl2, 1% Triton X-100, and 0.1 mg/ml bacitracin. The AChE activity of cell lysates and media was assayed by the colorimetric method of Ellman et al. (6). Enzyme samples (10 μl) were added to 0.2 ml of Ellman assay medium, and the reaction was monitored at 414 nm at 20-s intervals over a period of 5 min, using a Multiskan RC microplate reader (Labsystems, Helsinki, Finland).

RNA Preparation and Northern Blot Hybridization—Two days after transfection, COS cells and the RNA were extracted in the RNA-PLUS buffer according to the manufacturer's instructions (Quantum, Illkirch, France). RNA (6 μg/sample) was analyzed by electrophoresis in a 1.5% formaldehyde agarose gel and transferred to a nitrocellulose membrane. The membrane was prehybridized with the Emilson-Kurland buffer (900 mM NaCl, 90 mM EDTA, 1% SDS, 1 mg/ml polyvinylpyrrolidone, 1 mg/ml Ficoll, salmon sperm DNA) at 60 °C for 1 h. It was then hybridized with a 32P-labeled DNA probe corresponding to the signal peptide coding sequence, which is common to all constructs, by incubation at 60 °C for 1 h and then slowly decreasing the temperature from 60 to 30 °C overnight. The membrane was washed twice in 6× saline sodium citrate at room temperature, twice in 3× saline sodium citrate containing 0.5% SDS at 50 °C (30 min for each wash), and exposed to a Fuji imaging plate. The intensity of the bands was quantified using the TINA program.

Metabolic Labeling—Two days after transfection, COS cells were preincubated for 45 min in Dulbecco's modified Eagle’s medium without cysteine and methionine and labeled for 30 min with 50–100 μCi/ml of [35S]methionine-cysteine (Amersham Pharmacia Biotech). After labeling, the cells were rinsed with phosphate-buffered saline and chased in nonradioactive containing medium. At various chase times, the culture medium was collected, and the cells were homogenized with a Potter homogenizer in a buffer containing 20 mM borate, pH 9, 1 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1 mg/ml bovine serum albumin, and a protease inhibitor mixture (reference P-8340, Sigma), and centrifuged at 15,000 rpm for 30 min.

The cell extract and the medium were incubated overnight at 8 °C on a roller with a 1:300 dilution of a mixture of four anti-Bungarus AChE monoclonal antibodies (bunga-15, bunga-46, bunga-72, and bunga-82; (7)) in the case of Bungarus AChE and with a 1:300 dilution of the polyclonal anti-rat AChE rabbit antisera A63 (8) in the case of the rat and chimeric AChEs, then mixed with 20 μl of a suspension of protein A-Sepharose 4B (Sigma), and incubated for 3 h at 4 °C. AChE activity was totally immunoprecipitated by the anti-rat AChE A63 polyclonal antibody (8) in the case of the Bungarus/rat chimera (bR2) and more than 70% in the case of the rat/Bungarus chimera (bR2). In the latter case, addition of the four monoclonal anti-Bungarus AChE antibodies did not improve immunoprecipitation, suggesting that these antibodies recognize the N-terminal part of Bungarus AChE that is not included in this chimera. The beads were rinsed three times with extraction buffer, and the immunoprecipitated AChE sample was analyzed by electrophoresis in a reducing SDS-PAGE gel, using a Fuji image analyzer (BAS 1000). Each band was quantified using the TINA program. The quantification was normalized according to the numbers of methionine and cysteine residues contained in each protein.

CAT Expression and Western Blots—Expression of the CAT and neomycin resistance genes was monitored by Western blotting, using mouse anti-CAT (Amersham Pharmacia Biotech) or mouse anti-neomycin monoclonal antibodies (PEG 5′ Prime, Inc. Boulder, CO). The cells were extracted in buffer containing 50 mM Tris-HCl, pH 7, 10 mM MgCl2, 1% Triton X-100, and 0.1 mg/ml bacitracin, 2 days after transfection. Extracts were treated in a denaturing and reducing buffer and applied to a 12% polyacrylamide gel for SDS-PAGE. After immunoblotting, the protein bands were labeled with primary anti-CAT and anti-neomycin resistance antibodies and then labeled with a secondary antibody cou-
Expression Vectors for Secreted Dimeric AChEs—Fig. 1 shows the constructs that we used to compare the biosynthesis of Torpedo, Bungarus, rat AChE and various chimeras between these enzymes. We designate the different constructs by the combinations of fragments that they express.

To examine the expression of these proteins under conditions that would be as similar as possible to each other, we introduced several modifications to the initial cDNA sequences. The 3' and 5' noncoding regions of rat, Bungarus, and Torpedo cDNA were removed, and all the cDNAs were inserted at the same site in the expression vector pcDNA3. To avoid differences in post-translational processing, we removed the alternatively spliced C-terminal peptides and replaced them by short peptides containing a cysteine allowing the formation of disulfide-bonded dimers. All constructs therefore produced secreted AChE dimers. We chose to produce dimers because they are generally more stable than monomers, especially in the case of Torpedo (4, 9). Because the signal peptide that targets secreted proteins to the endoplasmic reticulum may influence the production of the protein, the rat and Torpedo peptide signals were replaced by that of Bungarus AChE. As shown in Fig. 2, this replacement induced a 2-fold increase in the production of rat AChE.

We made reciprocal chimeras between Bungarus and rat AChEs by combining N- and C-terminal fragments of the mature coding sequence. The vectors expressing Bungarus, rat, and chimeric AChEs therefore possessed the same initiation and termination sites and differed only by the coding sequence of the mature catalytic domain.

Expression of Rat, Bungarus, and Torpedo AChEs in COS and C2 Cells—To investigate the expression of rat, Bungarus, and Torpedo AChE, we transfected the plasmids into COS cells and C2 cells. We first examined the level of AChE activity in the cell extract and in the medium, after 3 days at 37 °C. Torpedo AChE was not produced in an active form at this temperature, as reported previously (4). Fig. 2 shows the relative yields of active AChE proteins, deduced by dividing the measured activities by their respective turnover rates: the production of active Bungarus enzyme (bB) was five or six times higher than that of rat enzyme (bR); nevertheless, in both cases more than 90% of the total activity was recovered in the supernatant (Fig. 2). When the cells were transferred to 27 °C, the Torpedo enzyme was active and was secreted; the proportion of secreted enzyme was similar to that of rat and Bungarus AChEs (not shown).

It appeared possible that overexpression in COS cells, because of the replication of the plasmids, was not favorable for the production of rat AChE. Alternatively, an efficient expression of rat AChE might require specific muscle factors. The mouse C2C12 cell line would be more appropriate for expression of rat AChE on both accounts, because these cells are myogenic and because they do not replicate plasmids containing an SV40 origin. As shown in Fig. 2, however, the expression
After transfection of bB and bR AChE constructs, COS cells were pulse-labeled with 75 μCi/ml of [35S]methionine and [35S]cysteine for 30 min and then chased up to 360 min. AChE was immunoprecipitated from cell lysates and media and analyzed by SDS-PAGE under reducing conditions.

FIG. 3. Pulse-chase kinetics of Bungarus and rat AChE in COS cells. After transfection of bB and bR AChE constructs, COS cells were pulse-labeled with 75 μCi/ml of [35S]methionine and [35S]cysteine for 30 min and then chased up to 360 min. AChE was immunoprecipitated from cell lysates and media and analyzed by SDS-PAGE under reducing conditions.

of Bungarus AChE was, in fact, even more favored compared with that of rat AChE than in COS cells (9-fold). This comparison of the two plasmids therefore showed that the higher expression of Bungarus AChE, relative to rat AChE, is linked to the coding sequence and is not strictly related to cell specificity, in agreement with our previous observations. Metabolic Labeling of Bungarus, Rat, and Torpedo AChE Proteins—To determine whether the totality of synthesized AChE protein becomes active or whether folding into the active conformation constitutes a limiting step in the biosynthetic pathway, we performed metabolic labeling of AChE in transfected COS cells. Two days after transfection, when the COS cells had reached a stable rate of AChE production, they were incubated with [35S]methionine-cysteine for 30 min and then chased with nonradioactive medium for various periods of time, up to 6 h. The labeled AChE protein was immunoprecipitated from the cell extract and from the culture medium. Analysis by SDS-PAGE showed that Bungarus AChE protein was produced approximately five or six times more than rat AChE protein (Fig. 3). We detected two major bands of AChE polypeptides, in the case of Bungarus and rat AChEs. This heterogeneity seems to result from glycosylation, because we obtained a single, faster migrating band after N-glycosidase treatment (not shown), as previously reported (10, 11). The secretion of active enzymes followed the same kinetics, and 90% of the total activity was recovered in the medium after 6 h of chase. The Torpedo AChE protein was produced at an intermediate level between rat and Bungarus AChE proteins. When the cells were maintained at 37 °C, however, Torpedo AChE remained inactive and was not secreted (not shown). These results show that the difference in the level of expression between rat AChE and Bungarus AChE may be explained by the synthesis of the corresponding polypeptides, suggesting that it is probably not related to the stability or to the folding of these enzymes.

Production and Folding of Torpedo AChE and Bungarus/Rat AChE Chimeras—The preceding results show that the origin of the difference between expression of rat and Bungarus AChEs resides in the production of the polypeptides and therefore in the primary sequence of the DNA, RNA, or protein. It may be linked to specific elements of these sequences: in an attempt to identify such elements, we constructed reciprocal Bungarus/rat (bB,Rb) and rat/Bungarus (bR,bB) chimeras (Fig. 1). Both chimeras were catalytically active. The catalytic rates of rat, Bungarus, and rat/Bungarus were, respectively, 70, 42, and 73% of that of Bungarus AChE. Both chimeras produced less active enzyme than either Bungarus or rat AChE, the level of Bungarus/rat AChE being particularly low (Fig. 4A).

We compared the synthesis of the chimeric and native proteins by metabolic labeling (Fig. 4B). As observed previously, the nonchimeric Bungarus and rat AChE proteins were stable and largely secreted into the medium over a 5-h chase period (70%). The chimeric rat/Bungarus protein was also stable during this period but was secreted to a much lesser degree. The Bungarus/rat protein was produced at a lower level and appeared to be less stable than the other three proteins; it was only barely detectable in the medium after the chase. At 3 days following transfection, more than 85% of the total activity was recovered in the culture medium in all cases, even in the case of the Bungarus/rat chimera (Fig. 4A). All these enzymes remained stable in the medium after secretion.

The ratios of active protein to total protein were calculated from AChE activities and [35S]labeling, so as to estimate the efficiency of folding, as shown in Table I. The resulting values are consistent with the idea that the totality of the natural Bungarus (bB) and rat (bR) enzymes acquires an active conformation, whereas only about half of the rat/Bungarus polypeptides and less than 5% of Bungarus/rat become correctly folded.

By analogy with Torpedo AChE, it appeared possible that the efficiency of folding of the chimeric enzymes might also be sensitive to temperature. We therefore transferred the COS cells to 27 °C for 4 days, under conditions that allow the folding of active Torpedo AChE. The activity of the Bungarus/rat chimera was increased about 3-fold at 27 °C, compared with 37 °C relative to the parent proteins. In contrast, the relative activity of the rat/Bungarus chimera remained essentially the same.

The fraction of labeled protein that was secreted after the 5-h chase closely reflects the fraction of active, correctly folded enzyme (Fig. 4B and Table I), in agreement with the fact that most of the active enzyme was recovered in the medium, after a period of 3 days (Fig. 4A and Table I). It thus appears that the acquisition of an active conformation is necessary and sufficient for secretion, in the case of AChE molecules from which the C-terminal peptides have been deleted, as studied here.

Although the labeled Bungarus/rat and Torpedo AChE proteins, which do not fold efficiently, were not recovered in the medium, they decreased in the cells during the 5-h chase period, indicating that they were degraded (Fig. 4B).

Steady State Level of AChE mRNA—We asked whether species differences in the synthesis of AChE proteins were related to translation of the polypeptides or to the levels of the corresponding mRNAs. We performed Northern blot analyses with a probe corresponding to the signal peptide from Bungarus (b), which is common to Torpedo (bT), Bungarus (bB), rat (bR), and chimeras (bR,bB,bR,bB). We used the 5.8 S ribosomal RNA to standardize the total amount of RNA loaded in Northern blots. To make sure that transfection efficiency was equivalent, we co-transfected each construction with a vector expressing a foreign protein. For convenience, we chose the Qn/stop protein as control (5), because the constructs and the probes were immediately available, this protein is well expressed in our system, and it does not interact with our enzymes. In every case, the levels of Qn/stop mRNAs were comparable, in parallel transfections; RNA from untransfected COS cells produced no detectable signal. As shown in Fig. 5, we observed a single band of the same size for each of the five AChE constructs, but with an increasing intensity in the order: bR < bR,bB, bT = bB = bB,bR.

The quantification of mRNAs encoding the Bungarus, rat, and rat/Bungarus enzymes showed that their levels were proportional to the incorporation of radioactive precursors into the corresponding proteins. The amounts of synthesized Torpedo and Bungarus/rat AChEs were lower than expected on the basis of this proportionality, possibly because these proteins, which were largely misfolded, were aggregated and were not...
Fig. 4. Expression of natural and chimeric AChEs in COS cells. A, AChE activity accumulated in the cells (hatched bars) and in the medium (white bars), 3 days after transfection. The relative amounts of active AChE were deduced from activities as in Fig. 2. B, metabolic labeling and chase of AChE. Cells were pulse-labeled for 30 min and analyzed immediately or after a 5-h chase period. The hatched and white bars correspond to the cell extract and medium, respectively. These data represent the means of two (A) or three (B) independent experiments, which did not differ by more than 20%.

Table 1

| Construct     | Secretion of active AChE | Secretion of AChE protein | Folding efficiency |
|---------------|---------------------------|---------------------------|--------------------|
| Bungarus (bB) | 95                        | 85                        | 100                |
| Rat (bR)      | 90                        | 70                        | 90                 |
| Torpedo (bT) | 0                         | 0                         | 0                  |
| Bungarus/rat (bB1R1) | 85              | 3                         | 3                  |
| Rat/Bungarus (bR1bB) | 90          | 20                        | 45                 |

Efficiently solubilized in our experimental conditions. A comparison of the two chimeras showed that the 5′ region of the coding sequence plays a major role in the determination of mRNA levels.

Effect of Actinomycin D and Cycloheximide on the Level of AChE mRNA—We analyzed the effect of actinomycin D, which blocks transcription of mRNAs, on the levels of Bungarus (bB) and rat (bR) transcripts in transfected COS cells. Actinomycin D did not induce a significant decrease in the levels of AChE mRNAs after 6 h, indicating that they were relatively stable (Fig. 6). Thus, the difference between the steady state levels of rat and Bungarus AChE mRNA does not seem to reflect mRNA stability but more probably the rate of transcription.

This is consistent with the effect of cycloheximide, which blocks translation and the production of unstable nucleases that degrade RNA (12). Two days after transfection, COS cells were treated for 6 h with cycloheximide. As shown in Fig. 5, we observed that cycloheximide increased the level of mRNA by the same factor for the rat, Bungarus, and Torpedo AChE constructs, as well as for chimeric Bungarus/rat and rat/Bungarus constructs, supporting the idea that the differences observed in the levels of the three mRNA species did not result from different degradation rates. The fact that the differences were maintained in the presence of cycloheximide indicated that they are not related to the translation process.

Injection of DNA or mRNA into Xenopus Oocytes—In Xenopus oocytes, the transcription rate should influence the efficiency of expression from cDNA, but not expression from mRNA. In contrast, RNA stability and translation efficiency should be equivalent in both cases. To determine whether the transcription rates of rat AChE and Bungarus AChE cDNA were different, we injected Xenopus oocytes into the nucleus with the pcDNA3/bR and pcDNA3/bB vectors or into the cytoplasm with mRNAs synthesized in vitro from these vectors.

The ratio of activities produced in the two cases should reveal whether the rate of transcription is different for the two constructs. As shown in Fig. 7, injection of mRNA produced a little more Bungarus than rat AChE. However, a much more dramatic difference was observed after injection of cDNA, which produced about 30 times more Bungarus AChE than rat AChE. These observations are in agreement with the previous results and suggest that transcription represents the major limiting factor for expression from cDNA.
factor for expression of rat AChE, compared with Bungarus AChE, in Xenopus oocytes.

The Difference in Bungarus and Rat Transcripts Is Not Related to Translation—To examine whether translation of AChE proteins may affect the levels of mRNAs, we inverted the coding sequences, in the pcDNA3/bR and pcDNA3/bB vectors. RNA produced from these constructs was analyzed by Northern blotting, using the same double-stranded DNA probe, corresponding to the signal peptide, as in Fig. 5. The level of Bungarus inverted transcript was 4–5-fold higher than that of rat inverted transcript, a difference similar to that observed in the illustrated experiment and in several others. AChE protein was deduced from activity, by dividing with the $k_{cat}$ values.

DISCUSSION

Folding Is Limiting for Torpedo and Chimeric AChEs, but Not for Rat and Bungarus AChEs—In vertebrates, AChE is encoded by a single gene; in some species, the catalytic domain may be followed by several C-terminal peptides, generated by alternative splicing. These various C-terminal peptides do not influence the catalytic activity of the enzyme but define its post-translational maturation and its association with structural proteins, thus allowing different types of anchoring to the plasma membrane or the extracellular matrix (14, 15). To study the structure and the function of the enzyme, AChE has been produced in several expression systems, including Escherichia coli, Pichia, Baculovirus-infected and plasmid-transfected insect cells and transiently and permanently transfected mammalian cells (HEK, COS, and RBL). In the case of RBL cells, the C-terminal peptides were found to influence considerably the fate of the synthesized protein (16). On the other hand, the production of active enzyme was found to vary markedly between AChEs from different vertebrate species, even when the proteins were limited to the catalytic domain by deletion of the C-terminal peptides, generating only soluble, secreted molecules (1). Some of these differences may be understood by considering the physical conditions pertaining to the normal biosynthesis of the enzyme and its intracellular properties; for example, the expression of active Torpedo AChE is highly temperature-sensitive so that its active conformation is not obtained at 37 °C and only partially at 27 °C (4), in agreement with the fact that Torpedo rays live in cold sea water. A more surprising finding was that the production of active AChE from Bungarus venom was about 20-fold higher than that of rat AChE in transiently transfected COS cells, using the same structures were predicted by the M-Fold software (13) to be equally stable in both cases and therefore did not appear likely to be responsible for the observed difference.

The 5′ region of the rat gene (R1) is predicted to form a loop that we tried to destabilize by replacing five Gs or Cs with As, without changing the protein sequence. This modification increased the production of rat AChE by about 30% in the case of the bR construct, doubling it in the case of a construct expressing the rat enzyme with its own signal peptide (rR). However, these mutations may affect not only the secondary structures but also binding sites for transcription factors.

The 5′ regions of the coding sequences of Bungarus (718 nucleotides) and Torpedo contain one potential SP1 site, whereas the corresponding rat region does not (Fig. 8). The presence of this site might therefore be related with the higher transcription of bB, bB1R2, and bT constructs, compared with bR and bR1B2. However, its suppression by a silent mutation did not modify the yields of active AChE, so that it does not seem to represent a functional cis-acting element. Similarly, mutations in other G+C-rich motifs did not affect the levels of AChE expression.

Do the cis-Regulatory Elements Stimulate or Depress Expression, and Are They Necessarily Transcribed?—We compared the expression of the CAT gene, to which we added the DNA fragments bB1, rR1, or bR1, either before or after the polyaenylation site, in the pcDNA3 vector. Expression was monitored by Western blots of CAT and of the neomycin resistance protein, which is encoded in the same vector and was used as an internal standard (Fig. 9). We found that rR1 and bR1 had little or no effect, in either position, compared with a simple CAT construct. The bB1 fragment had no effect when placed after the polyaenylation site and increased the expression of CAT about 3-fold, when placed between the CAT gene and the polyaenylation site. This sequence therefore seems to exert a stimulating effect, but only when included in the transcript.

A Search for cis-Elements Controlling Transcription Rate—We tried to define cis-elements that might cause the difference between the rates of transcription of rat and Bungarus AChE cDNAs. Fig. 8 shows that the two sequences present considerable conservation. Both possess a high G+C content (65% for Bungarus and 60% for rat). Hypothetical secondary structures were predicted by the M-Fold software (13) to be equally stable in both cases and therefore did not appear likely to be responsible for the observed difference.

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vector with a cytomegalovirus promoter, both at 37 and 27 °C (1); a similar difference, although less marked, was observed in the yeast *P. pastoris*, under the alcohol-oxydase 1 promoter (2).

Using stably transfected S2 *Drosophila* cells, under the control of the actin promoter, as described in the case of *Drosophila* AChE (17), we also obtained about 20 times more Bungarus than rat AChE. Thus, the more efficient production of Bungarus versus rat AChE does not depend on the C-terminal peptide, on the cell type, or on the promoter.

Recent experiments showed that Bungarus AChE recovers its active conformation after renaturation more easily than *Torpedo* AChE. It was therefore tempting to relate the difference between Bungarus and rat enzymes to intrinsic properties of the proteins; the different yields of recombinant Bungarus and rat AChEs might reflect their capacity to acquire an active conformation or their intracellular stability. It was already known that the *Torpedo* enzyme cannot fold properly and acquire a catalytically active conformation at 37 °C.

In this study, we explored the biosynthetic steps that control the production of active AChE. We analyzed the expression of *Torpedo, Bungarus,* and rat AChEs, which present about 80% similarity in peptide sequence, as well as chimeras between Bungarus and rat enzymes. We deleted the alternative C-terminal peptides to avoid differences resulting from post-translational modifications. In addition, because *Torpedo* AChE appears to be more stable as a dimer than a monomer, we introduced a short C-terminal peptide containing a cysteine, allowing the formation of secreted disulfide-linked dimers. All enzymes were thus produced in the form of soluble dimers, avoiding differences arising from post-translational processing because of the C-terminal peptides that normally follow the catalytic domain. In addition to the rat and Bungarus constructs, we analyzed two complementary rat/Bungarus (*bR1B2*) and Bungarus/rat (*bB1R2*) chimeras, as well as a *Torpedo* construct.

We performed transient transfection of mammalian cells and *Xenopus* oocytes, using constructs that differed only in the region encoding the catalytic domain: the 5' and 3' ends of the coding region, corresponding to the secretion signal peptide and to the short C-terminal peptide were generally identical. The synthesis of AChE polypeptides was monitored by metabolic labeling and was compared with the production of active

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**FIG. 8.** Sequence comparison of the 5' coding regions of rat and *Bungarus* AChEs. The 5' regions of Bungarus (*bB*) and rat (*rR*) AChE genes were aligned according to GeneWorks. Identical nucleotides are shown in gray boxes, and represent 65% of the illustrated regions. Arrows indicate the limits of the 5' sequences encoding the signal peptides: note that we used the Bungarus sequence (b) instead of the rat sequence (r) in most of our constructs. The performed silent mutations are shown above the sequence for Bungarus and below the sequence for rat. Mutations that are less than 30 nucleotides apart were introduced together. The replacement of Gs and Cs by As in the 5' part of the rat sequence was designed to destabilize a possible loop. Other mutations created or suppressed selected GC motifs, as indicated by solid bars above and below the sequences; in particular, single mutations, located in the 3' region of the sequences, either add an SP1 site (GGCGGG) in *rR1* or remove it from *bB1* (mutations that remove or add an SP1 site are indicated in normal characters and italics, respectively).

**FIG. 9.** Effect of the rat and Bungarus 5' coding regions on CAT expression. The 5' coding regions of rat and Bungarus AChEs were inserted before or after the polyadenylation signal of a CAT construct, in the pcDNA3 vector, as indicated by positions A and B, respectively. The expression of CAT was quantified from Western blots and normalized to that of the neomycin resistance gene, with the expression obtained without an added DNA fragment being taken as 100%.
AChE. We found that folding is indeed a limiting factor in the case of the chimeras, particularly Bungarus/rat in which less than 5% of polypeptide chains achieve a correct folding. The folding of Torpedo AChE is clearly temperature-sensitive, because the protein was inactive when produced at 37 °C and partly active at 27 °C; we also observed a small effect for Bungarus/rat but not for the rat/Bungarus chimera. Within a period of a few hours, the correctly folded, active molecules were secreted, whereas inactive molecules remained trapped within the cells. The difference between the yields of rat and Bungarus enzymes, however, could not be explained by folding, which appeared equally efficient in both cases.

Existence of cis-Elements Stimulating Transcription within the Coding Sequence of Bungarus AChE: Orientation Independence and Localization in the Transcribed Region—The difference between rat and Bungarus AChE activity and protein was found to reflect the levels of bR and bB mRNAs. More generally, we observed a direct relationship between the production of protein and the levels of transcripts obtained by transcription of our different constructs. These results indicate that the coding sequence of AChE determines the level of steady state AChE mRNA, which in turn determines the amount of AChE protein expressed. The fact that the same difference could be observed in the presence of cycloheximide and in inverted constructs, in which the coding sequences were introduced in the noncoding orientation, confirmed that it is totally independent of protein synthesis.

The higher level of Bungarus versus rat transcripts did not appear to reflect a difference in their stability, as shown by actinomycin D and cycloheximide treatments. In the presence of actinomycin D, which blocks polymerase II, all transcripts were stable over a 6-h period. Cycloheximide blocks the synthesis of nucleases and thus stabilizes unstable mRNAs (12); it increased the levels of all transcripts to the same degree. This increase suggests that these different transcripts were degraded at the same rate in the absence of drug, a degradation that was probably suppressed by actinomycin D as well as by cycloheximide. In any case, our results do not indicate any difference in the stability of the different mRNAs. This finding does not contradict the observation that AChE mRNAs were found to be unstable in the myogenic C2C12 mouse cell line prior to fusion and were stabilized upon fusion into myotubes (18), because we removed the introns and untranslated regions, which probably control the stability of mRNAs.

We conclude that the coding sequences of rat and Bungarus AChEs contain cis-elements that influence the rate of transcription. This effect was observed in several expression systems, using different promoters: S2 Drosophila cells, with an actin promoter,2 and COS cells and Xenopus oocytes with the cytomegalovirus promoter, in the present work, as well as Pichia yeast cells with the alcohol-oxydase 1 promoter in a previous study (2). This shows that the effect is not due to a fortuitous interference between the coding sequences and the promoters or other parts of the vectors but rather reflects an intrinsic property of the coding sequences themselves. The rat and Bungarus coding sequences are strongly homologous, presenting similar G+C contents (60 and 65%, respectively) and about 65% identity. This situation appears, therefore, extremely favorable for identifying the elements that control transcription.

A comparison of the bR,B2 and bR,R2 chimeras with the bR and bB constructs showed that the 5' region exerts a stronger effect than the 3' region on transcription. This includes the sequence encoding the N-terminal region of the mature protein and also the sequence encoding the signal peptide, because its replacement by that of Bungarus in the bR construct increased the production of rat AChE by about 2-fold. It seems, therefore, that this regulation involves several cis-elements.

It is possible that the observed effects depend on the secondary structures of either DNA or RNA. For example, the presence of a G+C-rich secondary structure in the 5' coding region of the human AChE gene has been proposed to exert an attenuating effect on transcription (19). However, mutations that were designed to disrupt a potential G+C-rich hairpin in the rat sequence were not conclusive, because they slightly increased the production of rat AChE, but not up to the level of Bungarus AChE, and their effect might reflect the disruption of sequence motifs exerting a negative control on transcription. If transcription was hindered by secondary structures, this effect would be expected to be largely independent of the polymerase; we compared the rates of in vitro transcription of rat and Bungarus templates with T7 RNA polymerase and found no difference (results not shown).

We observed that Bungarus and Torpedo sequences, but not the rat sequence, contained an Sp1 site in the 5' region. Because Sp1 or Sp1-like factors are ubiquitous, existing in all eucaryotic cells, these motifs appeared to be interesting candidates for explaining the higher rate of transcription of the Bungarus and Torpedo constructs. However, silent mutations disrupting such sites did not modify transcription. Of course, this lack of effect does not rule out the possible existence of other functional sites.

The fact that the levels of transcripts were maintained when the sequences were inverted shows that the influence of cis-elements on transcription is insensitive to their orientation and to their precise distance from the initiation site, suggesting either an enhancer or silencer effect or the presence of pause-causing elements. Using several CAT constructs, in which 5' regions of rat and Bungarus were inserted either before or after the polyadenylation site, we asked whether their regulatory influence was positive or negative, and whether their transcription was necessary. We found that the regulatory sequences of rat had no influence, whereas the Bungarus sequences increased expression but only when included in the transcribed region. This seems to exclude both a classical enhancer mechanism and a negative effect on transcription because of pauses. We do not know whether these conclusions can be generalized to other conditions (gene, promoter, localization and orientation of insert, position within the transcribed or untranscribed regions) and whether the stimulating effect can be transferred to other genes.

In most studies, the analysis of cis-elements controlling transcription is focused on the 5' genomic region, preceding the transcription initiation site. This is a natural consequence of the experimental strategy that uses the expression of a reporter gene, driven by more or less extended upstream regions of the studied gene. It is well known, however, that intragenic cis-elements controlling transcription also exist in introns and in the 3' region of genes. For example, the presence of an N-box element in the first intron of the rat AChE gene has been recently shown to ensure synapse-specific expression in muscle fibers in vivo (20). Deletion of the homologous intron of the mouse AChE gene did not affect the promoter activity in transfected fibroblasts (21) but reduced expression in the myogenic C2 cells (22).

The role of the coding sequence in gene regulation has been less often considered, probably because it is not easy to find appropriate experimental systems. The presence of regulatory elements within the coding sequence has been reported in a few cases, and it has even been suggested that intragenic elements may play a major role in the regulation of transcription. The fact that α globin is expressed about 10-fold more than β globin
is an excellent example. The two genes are similar in their exon/intron organization but differ markedly in their G+C content. The construction of chimeras led to the conclusion that intragenic cis-elements control the transcription level (23). These elements include Cp1, SP1, and α-IRP sites within a CpG island (24). However, these elements appeared to be exclusively intronic, in contrast to the present study, in which we focused on the effect of the coding exons. The involvement of both intronic and exonic modulatory sites has been documented in the case of the dihydrofolate reductase promoter (25).

It was particularly instructive to compare the expression of Torpedo, rat, Bungarus, and chimeric AChEs, because they show considerable differences despite their high degree of identity, at the DNA and peptide levels. Our analysis showed that the coding and primary sequences of a protein, respectively, contribute to transcriptional regulation by a novel mechanism and condition folding through self compatibility, as shown in chimeras. We found that the coding sequence of Bungarus AChE contains elements that stimulate transcription and may represent an evolutionary adaptation to the abundant expression of the snake enzyme in venom glands, which might not be supported by the structure of the rat AChE gene. It would be extremely valuable to identify such intragenic elements and elucidate their mode of action, because the same regulatory mechanisms may be of general importance for controlling the level of gene expression, in addition to classical promoters and enhancers. These effects might be useful to increase the production of recombinant proteins.

**Acknowledgments**—We thank Dr. Muriel Amar for injecting Xenopus oocytes, Celine Calvet for participation in some experiments, Dr. Susan Joyce for advice with the Northern blot experiments, Monique Lambergeon for providing the C2 cells, Dr. Olivier Jean-Jean for the gift of a CAT vector, and Drs. Suzanne Bon, Claire Legay, Olivier Bensaude, Marc Dreyfus, and Israël Silman for helpful discussions.

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