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MOLECULAR CHARACTERIZATION OF S1, A MEMBER OF THE MAMMALIAN ELONGATION FACTOR-1 ALPHA GENE FAMILY

by

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Abstract

Elongation factor-1 alpha (EF-1α) is a ubiquitous, highly conserved protein, that functions in peptide elongation during mRNA translation. A cDNA, S1, was isolated in rat; its predicted amino acid sequence shares high similarity (92.6%) with mouse and human EF-1α. The principal objective of this thesis is the molecular characterization of S1, a member of the mammalian EF-1α gene family. We have cloned an EF-1α cDNA in rat, and showed that rat S1 and rat EF-1α are two different mRNAs that share extensive sequence similarity in the coding region, but differ considerably in their noncoding regions. The 3' noncoding regions of S1 and EF-1α were subcloned into the pGEM3z plasmid and cRNA probes were synthesized using an in vitro transcription method. Northern analysis and RNase protection assays have shown that S1 mRNA can only be detected in rat and mouse brain, heart, and muscle, whereas EF-1α mRNA can be detected in all tissues. These results were confirmed by non-radioactive in situ hybridization. A strong S1-positive signal was detected in the hippocampus, cerebral cortex, medulla, and cerebellum of rat brain, whereas liver sections were negative. Certain cells, such as Purkinje neurons in the cerebellum, and motor neurons of the lower brain stem, were S1-positive. On the other hand, EF-1α mRNA was detected in all cell types that we have examined so far. These results prompted us to hypothesize that S1 is a terminal differentiation-specific EF-1α isoform, whose expression is limited to permanently growth-arrested cells. We tested this hypothesis by investigating the expression of S1 and EF-1α during rat development. S1 upregulation correlates with the terminal differentiation process during in vivo neurogenesis and myogenesis. Furthermore, S1 expression is dependent upon the formation of multinucleated myotubes during myogenesis in culture. EF-1α mRNA levels are down-regulated in the same period in S1-positive tissues, but stay relatively steady in liver. Its mRNA was detected in all cell lines that we have studied. Both S1 and EF-1α mRNA levels remain unchanged during the aging process in rat. We have cloned cDNAs for human and mouse homologues of rat S1. As for EF-1α, the S1 putative protein has been totally conserved among these three species. This suggests that the changes in the primary amino acid structure between S1 and EF-1α are important as they have been conserved during mammalian evolution. Taken together, our studies demonstrate that mammalian species contain a tissue-specific EF-1α isoform, whose expression is regulated in a postterminal-differentiation manner during brain, heart, and muscle development. They also intimate a novel mechanism by which protein synthesis may be regulated at the elongation step in permanently-growth arrested, long-lived cells such as neurons, and myocytes. This is, to our knowledge, the first demonstration of an altered protein synthesis factor whose expression is restricted to certain cell types in mammalian species.

Résumé

Le facteur d'élongation-1 alpha (EF-1α) est une protéine impliquée dans l'élongation de la chaîne peptidique lors de la traduction. Un ADNc, S1, a été isolé chez le rat et la séquence prédite en acides aminés est similaire à 92.6% à celle de EF-1α de la souris et de l'humain. Le but de cette thèse est la caractérisation moléculaire de S1, un nouveau membre de la famille génique de EF-1α chez les mammifères. Nous avons cloné l'ADNc qui code pour EF-1α chez le rat et démontré que S1 et EF-1α du rat sont deux différents ARNms qui ont une forte similarité de séquence dans la région codante, mais qui diffèrent considérablement dans les régions noncodantes. La région 3' noncodante de l'ADNc de S1 et de EF-1α a été sous-clonée dans le vecteur pGEM3Z afin de produire des sondes d'ARNc de ces régions, par la méthode de transcription in vitro. Des analyses Northern et de protection à la RNase ont démontré que l'ARNm de S1 est seulement exprimé dans le cerveau, le coeur et le muscle squelettique du rat et de la souris, tandis que l'ARNm de EF-1α est exprimé dans tout les tissus qui ont été étudiés jusqu'à maintenant. Ces résultats ont été confirmés par la technique de l'hybridation in situ non-radioactive. Aucun signal n'a été détecté dans des sections de foie de rat hybridées avec la sonde S1. Toutefois, un signal intense a été détecté dans l'hippocampe, le cortex cérébral, le cervelet, ainsi que la médulla du cerveau de rat. Certaines cellules telles les neurones de Purkinje dans le cervelet et les neurones moteurs dans la médulla sont positives pour l'ARNm de S1. L'ARNm de EF-1α a été détecté dans toutes les cellules que nous avons étudiées jusqu'à présent. En fonction de ces résultats, nous avons émis l'hypothèse que S1 est un isoforme de EF-1α dont l'expression est spécifique au cellules qui sont dans un état de différentiation terminale. Nous avons testé cette hypothèse par l'étude de l'expression de S1 et de EF-1α au cours du développement chez le rat. L'ARNm de S1 a été détecté seulement à la fin de la neurogenèse et de la myogenèse in vivo, avec un niveau d'expression qui augmente jusqu'au début de la vie post-natale. L'expression de S1 est dépendante de la formation de myotubes au cours de la myogenèse en culture, tandis qu'aucun signal n'a été détecté dans des lignées cellulaires non-myogeniques. Le niveau de l'ARNm de EF-1α diminue au cours de la neurogenèse et de la myogenèse, mais reste stable dans le foie, qui est un tissu qui n'exprime pas S1. L'ARNm de EF-1α a été détecté dans tout les lignées cellulaires que nous avons testé. Le niveau de l'ARNm de S1 et de EF-1α reste stable au cours du vieillissement du cerveau, du coeur, et du foie chez le rat. Nous avons cloné l'ADNc de S1 de la souris et de l'humain. La séquence en acides aminés prédite est totalement conservée entre S1 du rat, de la souris et de l'humain. Ces résultats suggèrent que les substitutions d'acide aminé entre la protéine S1 et EF-1α sont importantes car elles ont été conservées au cours de l'évolution chez les mammiferes. Dans l'ensemble, ces résultats démontrent que les mammifères ont un gène qui code pour un isoforme de EF-1α dont l'expression est dépendante de la différentiation terminale des cellules du cerveau, du coeur, et du muscle squelettique. En plus, ils suggèrent un nouveau mécanisme par lequel la synthèse de protéine est régulée au cours de l'élongation de la chaîne peptidique chez les cellules qui sont post-mitotiques, et qui ont une longue période de vie, tel les neurones, les cardiomyocytes et les myocytes.

PREFACE TO THE THESIS

The thesis presented herein is written in the form of original papers. The provision from the Thesis Guidelines reads as follows: "The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text, or duplicated published text (see below), of an original paper, or papers. In those cases the thesis must still conform to all other requirements explained in Guidelines Concerning Thesis Preparation. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g. appendices) to allow a clear and precise judgment to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction, and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interest of cohesion."

"It is acceptable for theses to include as chapters authentic copies of papers already published, provided these are duplicated clearly on regulation thesis stationary and bound as an integral part of the thesis. Photographs or other materials which do not duplicate well must be included in their original form. In such instances, connecting texts are mandatory and supplementary explanatory material is usually necessary."

As a result, four original papers are presented here, all of which are either published or submitted for publication. Chapters 2 to 5, which contain the manuscripts, include abstracts, introductions, materials and methods, results, and discussions as they appear in the literature or as submitted for publication. Moreover, because of space limitation in the Journals, certain figures and tables are included to complement the chapters. These figures and tables are depicted as "annexes." Connecting texts have been inserted between chapters to provide continuity. In accordance with McGill University's thesis guidelines,

this thesis also includes a general abstract (résumé), an introduction (review of the literature), and a general discussion.

The experimental work described in chapters 2 to 5 has been published or submitted for publication as follows:

Chapter 2: **Lee, S., Francoeur, A.M., Liu, S., and Wang, E.** (1992) Tissue-specific expression in mammalian brain, heart, and muscle of S1, a member of the elongation factor-1 alpha gene family. J. Biol. Chem., 267, 24064-24068.

Chapter 3: **Lee, S., Stollar, E., and Wang, E.** (1993). Localization of S1 and elongation factor-1α mRNA in rat brain and liver by non-radioactive in situ hybridization. J. Histochem. Cytochem., 41, 1095-1098.

Chapter 4: **Lee, S., Wolfraim, L. A., and Wang, E.** (1993). Differential expression of S1 and elongation factor-1 alpha during rat development. J. Biol. Chem. , 268, 24453-24459.

Chapter 5: **Lee, S., Ann, D. K., and Wang, E.** (1993). Cloning and characterization of cDNAs coding for mouse and human homologues of rat S1. Gene (Submitted for publication)

The conception, technical realization, and presentation of these projects are entirely mine. The published and submitted manuscripts, including the figures, presented in chapters 2 to 5 have been written and drawn by myself. Dr. Anne-Michele Francoeur and Dr. Sha Liu helped in isolating RNA of figures 2A, and 3 of chapter 2. Eva Stollar contributed in tissue sectioning in chapter 3. Dr. Lawrence A. Wolfraim contributed in correcting the English of chapter 4. Dr. David K. Ann contributed in sequencing

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TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

LIST OF ABBREVIATIONS

bp	: Base pair
cDNA	: Complementary DNA
CH_3	: Methyl group
cRNA	: Complementary RNA
DIG:	: Digoxygenin
DM:	: Differentiation medium
DMEM:	: Dulbecco's modified Eagle's medium
EF	: Elongation factor
FCS	: Fetal calf serum
GPEA	: Glycerylphosphoethanolamine
HS	: Horse serum
IF	: Initiation factor
kb	: Kilobase
kDa	: Kilo-Dalton
mRNA	: Messenger RNA
rRNA	: Ribosomal RNA
tRNA	: Transfer RNA
NRK	: Normal rat kidney
PIPES	: 1, 4-piperazinediethanesulfonic acid
PM	: Proliferating medium
PO_4-	: Phosphorylation
RF	: Release factor
SDS	: Sodium dodecyl sulfate
UTR	: Untranslated region

CHAPTER 1

LITERATURE REVIEW

1. Introduction

The number of elderly people in the province of Quebec, both in an absolute number as well as percentage with reference to total population, is growing at an ever increasing rate. It is estimated that 40% of the North American population will be in the 65 years and older category by the start of the next century. These statistics are alarming considering the economical and medical impacts of such an aged population, particularly when governments are desperately trying to equilibrate their budgets. While the medical cost of the elderly population will be soaring in the next few years, it is important to point out that perhaps an even greater cost to society is the steady decline in many physiological and mental processes that surface during human aging (Fies and Crapo, 1981). This phenomenon emphasizes the importance of directing research efforts toward preventing the causes rather than the effects of aging.

Human life expectancy has augmented dramatically in the past few centuries. This is primarily the result of the elimination of environmental hazards to which primitive cultures were subjected. In 1993, we can expect to live till we are about 75 years old. Yet, the increase in the number of years we are living does have a downfall (Marsh, 1989). A multitude of age-related diseases have emerged in the last century, such as cancers, heart failures, and debilitating neurodegenerative disorders including Alzheimer's and Parkinson's diseases. Of course, there is an important social and economic impact in treating and dealing with such illnesses and most of the research enterprise has focused on curing these major diseases (Marsh, 1989). Yet, once we have found ways to cure these diseases, we will probably start uncovering a wide range of new age-related health problems. Whereas the last century has seen an increase in the number of years we are living, the challenge for the near future will be to better the quality of these years.

There is a wide variety of conflicting theories of aging (Moment, 1982; Cutler, 1991). Most biologists do not accept the idea that aging has been a naturally selected trait during evolution (Cutler, 1984, Hayflick, 1987). Indeed, organisms will succumb to environmental hazards such as disease or predation before they die of old age. Kirkwood (1977, 1984) and Kirkland (1989) have suggested that aging is the result of the coupling of harmful mechanisms to early optimal developmental processes. Others have argued that cells undergo senescence and eventually die to prevent tumorigenesis, allowing organisms to survive until they reproduce (Sager, 1991; Dykhuisen, 1974; Cutler and Semsi, 1989). Whichever theory may prove right, most investigators agree that key genes are ultimately responsible for an organism's limited life span. This has led to the formulation of the longevity determinant gene hypothesis. Investigators have started to identify these genes by creating long-lived mutant strains of certain species, such as the nematode Caenorhabditis elegans (Friedman and Johnson, 1987; Johnson, 1988) and the fruit fly Drosophila melanogaster (Luckinbill et al., 1988; Arking and Dudas, 1989; Phillips et al., 1989; Seto et al., 1990). These mutants will eventually provide important clues in identifying genes that confer a longer life span.

How longevity genes exactly determine the life span of an organism is a highly debated question. Certain investigators have proposed that cellular aging is caused by the accumulation of errors in key macromolecules such as DNA, RNA, and proteins. These errors are due to a wide variety of environmental stresses, and a reduction in cells' repair mechanism capacity (Starke-Reed, 1989; Rattan, 1989; Crowley and Curtis, 1963; Harman, 1981; Medvedez, 1980; Cutler, 1983, Tomasoff et al., 1980). In this later case, longevity genes are considered to be a class of genes that either promote the presence of, or eliminate, damaging agents, therefore determining how many errors macromolecules suffer (Cutler, 1986; Rothstein, 1983). The second hypothesis considers senescence as being genetically programed, and is known as the program theory. While there is no doubt that there are well

defined genetically programmed mechanisms leading to apoptosis during early development (Bansal et al., 1991; Raff, 1992; Schwartz et al., 1990), the relation between developmentally regulated apoptosis and organism aging is for the less, unclear. In this case, longevity genes regulate the programmed cell death of certain cells, bringing about the death of the organism.

As in many biological problems, investigators have turned to cell culture systems to help clarify certain aspects of their work. The most widely used cellular model in aging is the human fibroblast. Hayflick (1965) showed that normal human fibroblasts have a finite replicative life span (Hayflick and Moorrhead, 1961; Hayflick, 1965). This restriction of proliferative capacity in culture has been interpreted as senescence at the cellular level (Goldstein, 1990; Warner and Wang, 1989). Moreover, the number of replications that a fibroblast can undergo in culture is directly proportional to the donor age. Fibroblasts obtained from a young donor will have a longer replicative life span in culture than fibroblasts isolated from an older donor (Goldstein and Harley, 1979; Rhome, 1981). Senescent fibroblasts display several altered characteristics, such as a decrease in transcription and translation activity, an increase in the number of abnormal nuclei, and chromosomal irregularities (Carlin et al., 1983; Cristofalo and Ragona, 1982; Nose and Okamoto, 1980; Okada and Dice, 1984; Dice, 1993; Dice, 1985). Interestingly, these altered characteristics are observed in certain aged organs such as brain, liver, and skeletal muscle (Crowley and Curtis, 1963; Dice, 1993; Dice, 1989; Lewis and Holliday, 1970; Richardson and Cheung, 1982). If the limited life span of fibroblasts in culture mirrors the overall aging phenomenon, then the molecules implicated in the control of permanent growth arrest could be key to the general understanding of aging (Smith, 1990; Warner et al., 1992). This is the rationale for studying mechanisms underlying the cellular growth arrest state. Over the past few years, several molecules directly implicated in the cell cycle

have been characterized. One of these cell-cycle related molecules, referred to as statin, was identified in 1985 by Dr. Wang's laboratory (Wang, 1985a, b).

Statin is a 57 kDa protein that can only be detected in the nucleus of nonproliferating cells, i.e., quiescent or senescent (Wang, 1985a, b). A logical step to start characterizing the cellular function of statin was to isolate its corresponding cDNA. To do so, a rat brain lambda gt11 cDNA library was screened with statin-specific S44 monoclonal antibody, and a cDNA, named S1, was isolated and characterized (Wang et al., 1989). S1 is a 1.7 kb cDNA that contains a long open reading frame of 463 amino acids, coding for the putative pS1 protein (Ann et al., 1991). The composition of the predicted amino acid sequence of S1 was compared to that of statin isolated from mouse 3T3 cells. This analysis indicated that statin and pS1 differ in their amino acid composition, predicted molecular weight, and pI. It is now clear that the two proteins are different, and that pS1 only shares an antigenic determinant with statin. This type of cloning artifact is not surprising, as there are several examples of spurious shared antigenic determinants among totally unrelated proteins.

pS1 does share significant sequence similarity with another protein, known as elongation factor-1 alpha (EF-1α). EF-1α is a ubiquitous and highly conserved protein that functions in peptide elongation during mRNA translation. The cloning of S1 implied that rats contained a second EF-1α gene never characterized so far. The identification of such an EF-1α isoform in a mammalian species is surprising, considering the substantial amount of work accomplished on EF-1α in the last thirty years. Relevant to the work in aging is the growing evidence relating EF-1α expression and activity to senescence in vivo and in culture. The isolation of the S1 cDNA has led to a new project in Dr. Wang's laboratory. Except for the nucleotide sequence of S1, absolutely no information was available on this novel gene. My thesis focused on the molecular characterization of S1 in mammals, in comparison to EF-1α.

To better situate S1 and EF-1α in the translational apparatus, the following sections provide a comprehensive review of the literature pertaining to protein synthesis mechanisms in eucaryotes, principally to EF-1α's role in translation. Section 2 is a summary of protein translation mechanisms in eucaryotes. This summary will situate EF-1α within the protein synthesis apparatus. The following sections are exclusively devoted to EF-1α. A historical review of the original experiments bringing about the identification EF-1α is the topic of the third section. It is followed by a survey of recent developments in the molecular cloning of EF-1α. Also included is a full review on EF-1α's role in development and aging. Section 7 describes the isolation and characterization of rat S1, concluding with a comparison between rat S1 and mammalian EF-1α. The last section is devoted to the principal research aims of this thesis.

2. Overview of translation mechanisms

2.1. Introduction

This section is a survey of the existing literature on protein synthesis mechanisms. Protein translation can be divided into three major steps: initiation, elongation, and termination. The reactions in each step of translation are promoted by soluble protein factors that interact with ribosomes, mRNA and aminoacyl-tRNAs. These steps will be treated separately in this section. However, the initiation step will be the principal issue, largely since aspects of the elongation step are extensively covered in the following sections. A general scheme of the different proteins and complexes involved in peptide synthesis is displayed in Figs. 1-4, and will often be referred to in the next few paragraphs. Also included in this section are examples of key regulatory mechanisms involved in the control pathway of protein synthesis.

2.2. Initiation

The initiation of translation is defined as the formation of the 80S initiation complex at the appropriate AUG start codon of a mRNA. A multitude of factors, known as initiation factors (eIFs), interplay to bring about the formation of the 80S complex. The importance of this step in translation has led to the suggestion that the regulation of protein synthesis is primarily mediated by the control of the initiation process. Therefore, it is not surprising that research efforts in the past decade have been concentrated on factors that are implicated in this step of translation. Eleven eIFs, themselves composed of several polypeptides, involved in four major events that lead to initiation, have been isolated and characterized in eucaryotic species (Figs. 1, 2). These events consist of: A) ribosomal dissociation into 40S and 60S subunits; B) met-tRNA binding to form the 43S preinitiation complex; C) mRNA binding to the 43S subunit; and D) junction of the 43S subunit with the 60S subunit to form the 80S initiation complex. These events and certain of the eIFs implicated will be treated separately in the next few paragraphs.

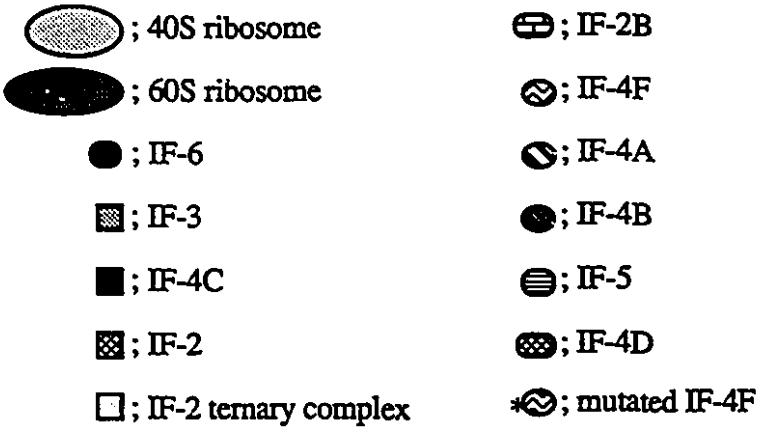
A) Dissociation of the 80S complex

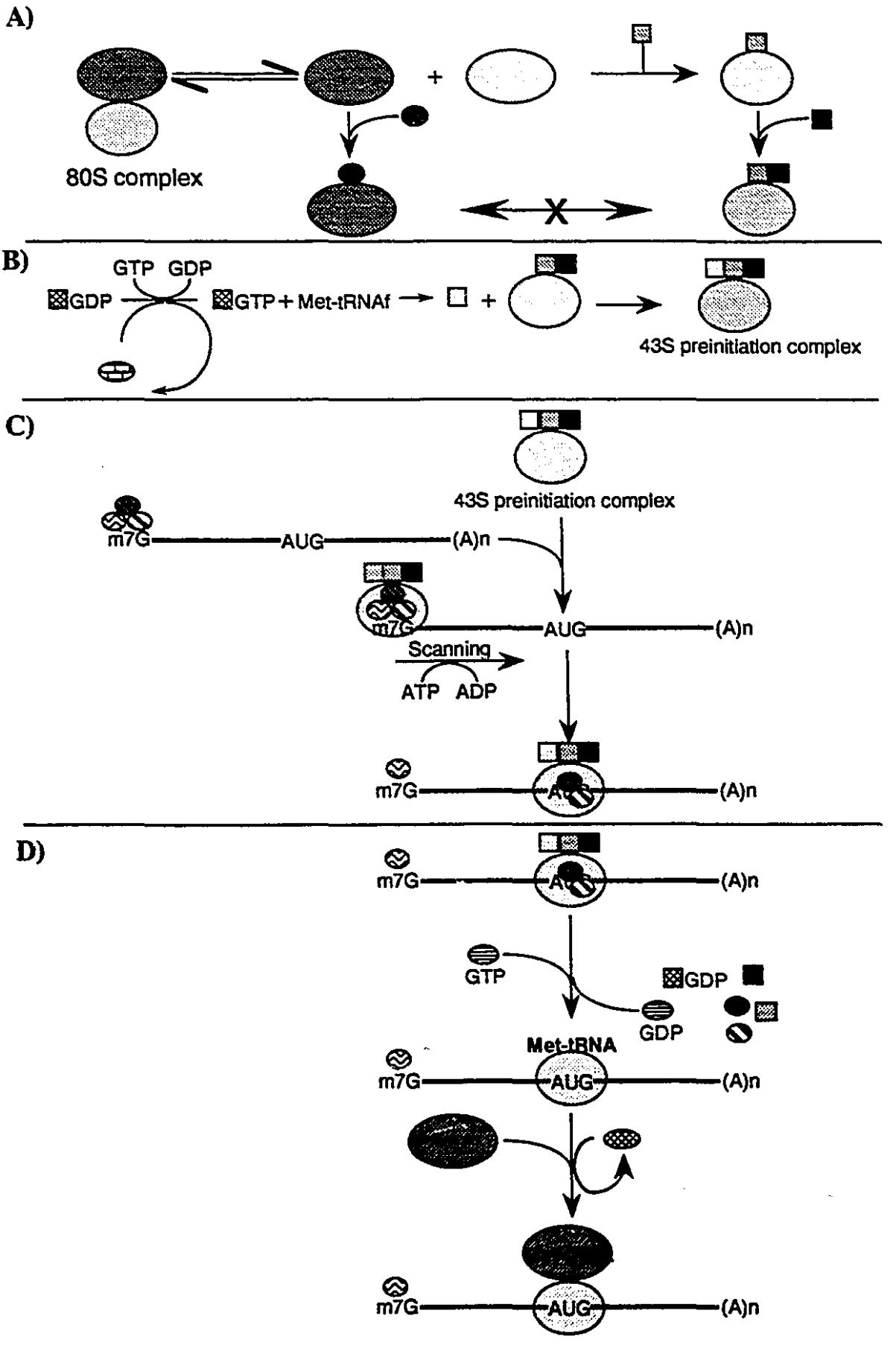
The first step consists of the dissociation of the 80S complex into its two subunits. The subunits, named 40S and 60S because of their sedimentation values, are themselves composed of various rRNAs and proteins (one molecule of 18S rRNA and about 30 proteins for the 40S subunit, and one molecule each of 5S, 5.8S and 28S rRNA and more then 45 proteins for the 60S subunits) (Nygard and Nilsson, 1990). The dissociation of the 80S complex is required to generate substantial amounts of free 40S subunit, crucial for the formation of the initiation complex. The dissociation of the 80S complex is catalyzed by three eIFs; eIF-3 and eIF-4C that interact with the 40S subunit, and eIF-6 that interacts with the 60S subunit (Benne and Hershey, 1978; Goumans et al., 1980; Raychaudhuri et al.,

Figure 1. Schematic representation of eucaryotic translation initiation apparatus.

A) Dissociation of the 80S complex. B) Formation of the 43S preinitiation complex. C) Binding of the 43S subunit to the mRNA. D) Formation of the 80S complex. This diagram is adapted from Merrick (1992), and Nygard and Nilsson (1990), with minor modifications. The description of the different steps is included in the text.

Figure legend:




A)
80S complex
B)
GTP GDP
GDP
GTP + Met-tRNAf
43S preinitiation complex
C)
43S preinitiation complex
m7G
AUG
(A)n
Scanning
ATP ADP
D)
GTP
GDP
Met-tRNA

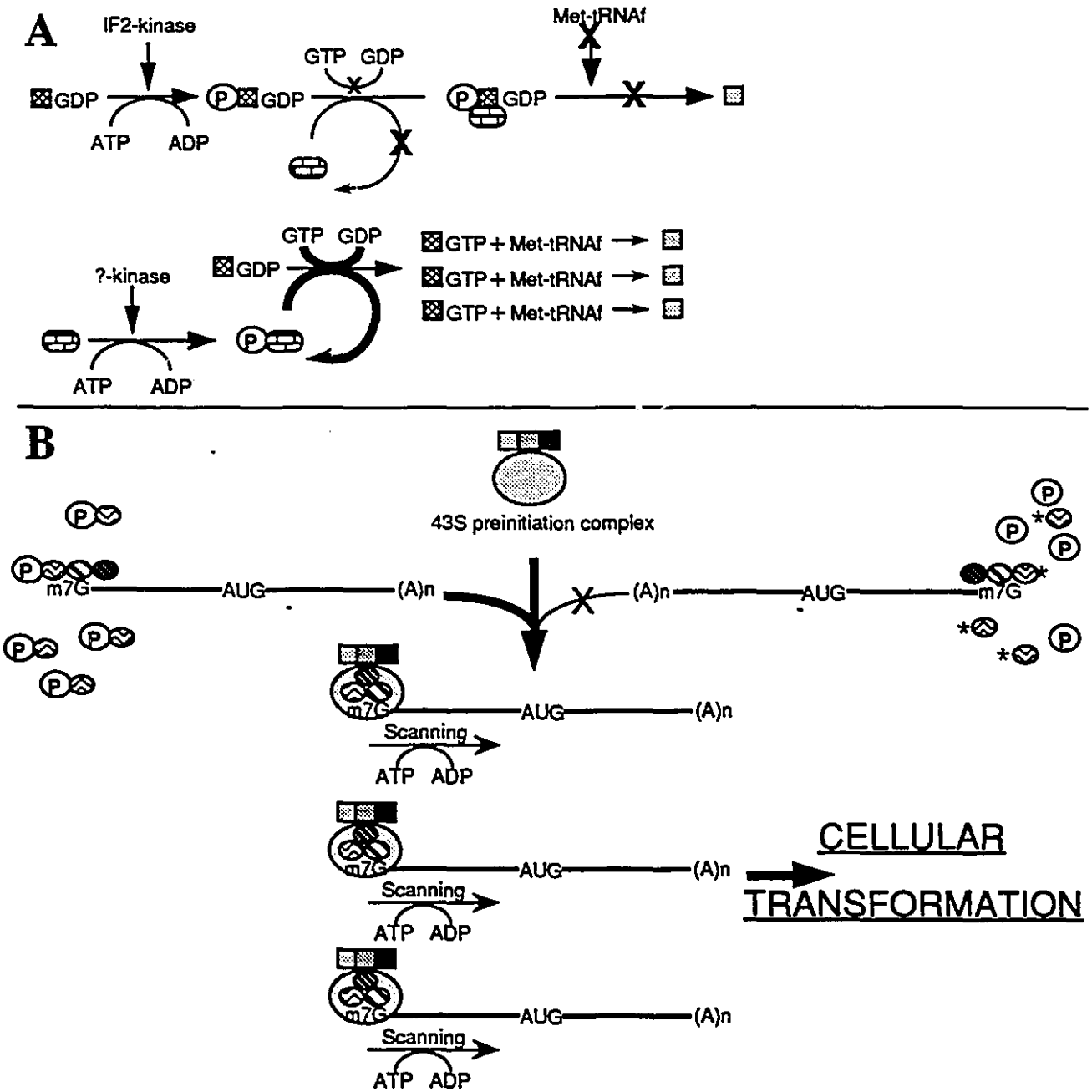
1984; Heufler et al., 1988). eIF-3, together with eIF-4C, is thought to play a role in the shifting of the equilibrium toward the dissociation of 80S, by preventing the 40S association with the 60S subunit. Likewise, eIF-6 is believed to prevent the formation of the 80S complex by binding the 60S subunit, although its exact cellular role in vivo is still widely debated. Even if levels of eIF-3 and eIF-4C are nearly equal to those of ribosomes, it is the 80S ribosomes that are routinely observed. Other not yet described elements are assumed to interact with the 80S ribosomes to favor its association. Nevertheless, there is always a small pool of free 40S subunit that is presumably created by the release of free subunits after the translation process.

B) Formation of the 43S preinitiation complex

This is a common step to the translation of all mRNA, and implicates the binding of an eIF-2-GTP-Met-tRNA ternary complex to a free 40S subunit to form the 43S preinitiation complex (Fig.1B). The primary function of eIF-2 is to bind to the initiator tRNA (Met-tRNA), in a GTP-dependent manner (Nygard et al., 1980). The formation of the ternary complex is important both to provide an aminoacyl-tRNA to the peptide site of the ribosome, and to select the appropriate AUG start codon. Once formed, the ternary complex binds to the 40S-eIF-3-eIF-4C subunit to form a particle with a sedimentation value of 43S (Benne and Hershey, 1978; Trachsel and Staehelin, 1979; Trachsel et al., 1977; Peterson et al., 1979a, b). The 43S subunit is then ready to bind mRNA, as will be discussed below. Once the GTP is hydrolyzed, the met-tRNA is released from the ternary complex and eIF-2 forms a binary complex with GDP (eIF-2-GDP) (Konieczny and Safer, 1983; Bagehi et al., 1982; Pain and Clemens, 1983). To form another ternary complex, the eIF-2-GDP binds to the guanosine exchange factor eIF-2B, which promotes the exchange of GDP to GTP on the eIF-2. Once bound to GTP, the eIF-2-GTP is then ready to form another ternary complex.

Figure 2. Schematic representation of regulation mechanisms at the initiation step of translation.

A) Regulation by phosphorylation of the formation of the eIF-2-GTP-met-tRNAf ternary complex. B) Regulation by phosphorylation of the eIF-4F cap-binding complex formation. The description of the regulation mechanisms is included in the text. The figure legend is the same as Fig. 1.


A
IF2-kinase
GDP
ATP
ADP
GTP GDP
Met-tRNAf
?-kinase
GTP + Met-tRNAf
GTP + Met-tRNAf
GTP + Met-tRNAf
B
43S preinitiation complex
m7G
AUG
(A)n
Scanning
ATP ADP
CELLULAR
TRANSFORMATION

This step of initiation is believed to be highly regulated, particularly by phosphorylation mechanisms. Many examples of a repression or activation of translation by phosphorylation of either the α subunit of eIF-2 or eIF-2B have been reported. In one case, eIF-2α specific kinases, referred to as eIF-2α kinase in Fig. 2A, are activated either by the deprivation of hemin (called the hemin regulated inhibitor) or by double-stranded RNA and interferon (called DAI or dsI), and phosphorylate the serine-51 residue of eIF-2α (Colthurst et al., 1987; Hershey, 1989; Pathak et al., 1988). The phosphorylation of Ser-51 is presumed to hinder the guanosine nucleotide exchange reaction by promoting a stronger association between eIF-2-GDP-eIF-2B, without the usual GDP to GTP exchange (Rowlands et al., 1988; Dholakia and Wahba, 1989; Pain, 1988). This in turns sequesters the eIF-2B, which is normally present in low quantity, thereby preventing the GDP to GTP exchange on other nonphosphorylated eIF-2-GDP. The binary complex is thus incapable of forming a ternary complex with met-tRNA, reducing the amount of 43S subunits that are necessary for the initiation of translation. That such mechanisms of repression of initiation by eIF-2α phosphorylation exist in vivo has been clearly demonstrated by several groups (Kaufman et al., 1989; Davies et al., 1989; Schneider and Shenk, 1987). The regulation of the ternary complex formation, as evidenced by eIF-2α phosphorylation, has been associated with a number of different physiological states other than heme deficiency and double-stranded RNA as described above, including heat shock, the presence of heavy metals, and deprivation of serum, amino acid, glucose, and insulin (Duncan and Hershey, 1984, 1985; Hurst et al., 1987; Scorsone et al., 1987; Towle et al., 1986). The second example involves the phosphorylation of eIF-2B itself (Dholakia et al., 1989; Dholakia and Wahba, 1989). Phosphorylation of eIF-2B augments its catalytic activity in the guanine nucleotide exchange reaction, and could therefore compete with the repression effect of eIF-2α phosphorylation (Dholakia et al., 1989). The interplay between phosphorylation of eIF-

2α and eIF-2B is presumed to be a crucial regulation point of protein synthesis in eucaryotes.

C) Binding of the 43S subunit to the mRNA.

There are two mainstream models regarding the binding of the 43S subunit to the mRNA: the internal initiation model and the scanning model (Hershey, 1991; Kozak, 1991, 1978, 1980, 1987). The first model proposes that the active 43S particle binds either directly to the start translation codon AUG, or somewhere upstream in the 5' UTR, followed by a scanning process. The second model suggests that the 43S subunit binds to the extreme 5' end (cap) of the mRNA, followed by a scan of the mRNA for the appropriate AUG codon. Virtually all evidence for the internal initiation model comes from the study of viral mRNA such as poliovirus (Pelletier and Sonenberg, 1986) and encephalomyocarditis virus (Jang and Wimme, 1990). One exception to this rule is a report of a cellular mRNA coding for the glucose-regulated protein 78/immunoglobulin heavy chain binding protein, which is translated using a cap-independent mechanism (Sarnow, 1989). It is conceivable that certain cellular mRNAs, with long 5' UTR and several AUG codons, such as human c-abl mRNA, are translated using an internal initiation mechanism (Bernards et al., 1987). However, the vast majority of cellular mRNAs are translated using the scanning mechanism, therefore I will center my attention on this specific model.

Three steps are necessary for the binding of the 43S subunit to the mRNA as predicted by the scanning model (Fig. 1C). The first step involves the binding of the eIF-4F, a protein composed of three subunits of molecular masses of 220, 45 and 24 kDa, to the 7-methyl-guanylic acid (cap) at the 5' terminus of the mRNA (Grifo et al., 1983, Helentjaris and Ehrenfeld, 1978; Sonenberg and Shatkin, 1977). The recognition of the cap structure is believed to be mediated by the alpha subunit, also known as eIF-4E (Edery et al., 1987; Sonenberg et al., 1979; Tahara et al., 1981; Webb et al., 1984). The second step

consists of eIF-4A and eIF-4B binding to eIF-4F-cap structure. The exact function of eIF-4B is unclear but it probably stimulates eIF-4A RNA dependent helicase activity (Abramson et al., 1987, 1988; Rozen et al., 1990). The third step involves the binding of the 43S subunit to the mRNA-eIF-4F-eIF4B-eIF-4A complex, probably by an interaction between eIF-4F and eIF-4B with eIF-3 on the 43S subunit (Hansen et al., 1982; Grifo et al., 1983). Once bound to the cap site, the 43S subunit starts scanning the mRNA for an appropriate AUG codon. The scanning of the 43S subunits is helped by the eIF-4A and eIF-4B helicase activity, that melts the 5' UTR of the mRNA until the appropriate AUG codon is reached.

This raises the question of how do the 43S subunits select for the appropriate AUG start codon? For 90% of eucaryotic mRNA, the answer appears fairly simple: the first AUG codon 3' from the cap structure is used to initiate translation (Kozak, 1978, 1980, 1987, 1989a, 1989b; Cigan et al., 1988a, b). As for the remaining 10% of mRNA, there is abundant controversy on precisely how the 43S subunit selects for the appropriate AUG codon. One explanation is the context in which the initiation codon occurs. A strong initiation codon is flanked by purines in positions -3 and +4 where +1 is the A in the AUG codon; this is known as the Kozak consensus sequence (Kozak, 1989a, b). When a scanning ribosome encounters an AUG with a weak consensus sequence, it may bypass this codon to the next one, which contains a stronger consensus sequence, thus initiating translation at the latter AUG codon. A second explanation is that the initiation at a particular AUG codon depends upon the kinetics by which the 43S subunit scans over an AUG, selecting for an AUG when scanning is slower. This idea has gained support in the last few years (Pelletier and Sonenberg, 1985; Cavener and Ray, 1991). It suggests that mRNA can form extensive secondary structure close to the AUG codon, capable of blocking, or at least slowing down, the migrating 43S subunit, permitting its interaction with the initiation

codon. Nevertheless, once the 43S interacts with the start codon, the 60S subunit binds to it to form the 80S complex initiation complex, as will be discussed below

There is evidence of phosphorylation of several eIFs implicated in the binding of the 43S initiation complex (Fig. 2B). The best-studied eIF in this group is the eIF-4F, particularly its alpha (also known as eIF-4E) subunit (Hershey, 1989; Tuazon et al., 1989). Phosphorylation of the Ser53 residue (Rychlik et al., 1987) of the cap-binding protein, eIF-4E, stimulates its in vitro and in vivo activity (Josh-Barve et al., 1990; Bonneau and Sonenberg, 1987). In vitro studies have revealed that eIF-4Fα binds to the 43S preinitiation complex in its phosphorylated form, but does not affect the binding of eIF-4Fα to the cap-site of the mRNA (Josh-Barve et al., 1990, Morley and Traugh, 1990, Panniers et al., 1985) The phosphorylation level of eIF-4Fα seems also to be important for its activity in vivo. Transfection of serum-starved quiescent NIH3T3 cells with the wild type form of eIF-4Fα induces initiation of DNA synthesis, whereas the mutant form of eIF-4Fα, with an Ala at position 53, cannot produce this effect by itself (Smith et al., 1990) (Fig. 2B). Probably the most interesting finding is that overexpression of eIF-4E in transfected mouse 3T3 cells induces cellular transformation (Lazaris-Karatzas et al., 1990; Smith et al., 1990). Again here, phosphorylation of the Ser53 seems important to obtain such a drastic change in metabolism, as the mutant form of eIF-4Fα (Ala at position 53) is incapable of transforming the same cells (Smith et al., 1990). As for the kinases that are responsible for the phosphorylation of eIF-4Fα, their identification is still somewhat unclear. Protein kinase C is one candidate, due to its capacity to phosphorylate eIF-4Fα at Ser53 in vitro (Morley and Traigh, 1990; Tuazon et al., 1989). Another kinase has been identified in vivo, but is not as yet well characterized (McMullin et al., 1988). Nevetheless, these results intimate a direct relationship between phosphorylation mechanisms, initiation of protein synthesis, and the control of cell growth.

D) Formation of the 80S complex

The fourth and last step of initiation of protein synthesis is the formation of the active 80S ribosomal complex (Fig. 1D). This occurs when the 43 subunit reaches an appropriate AUG start codon. At this time, GTP hydrolysis of the ternary complex (eIF-2-GTP-Met-tRNA-GTP) is catalyzed by the eIF-2 molecule, causing the release of the initiation factors from the 43S subunit (Benne and Hershey, 1978; Merrick, 1979; Raychaudhuri et al., 1985) . This event is triggered by eIF-5, a 125 kDa protein with a ribosome-dependent GTPase activity, and perhaps by its being complexed with eIF-4C (Ghosh et al., 1989; Peterson et al., 1979; Trachsel et al., 1977; Voorma et al., 1979). Although it has been shown that eIF-5 is over-phosphorylated during high protein synthesis activity, the exact regulatory function of such phosphorylation is not known (Hershey, 1989; Duncan and Hershey, 1987). The release of the eIFs is followed by the binding of the 60S subunit, an event that is thought to be catalyzed by eIF-4D, a polypeptide of 16 kDa (Benne and Hershey, 1978)). At this time, the 80S complex starts to migrate along the mRNA and incorporates new amino acids into the nascent polypeptide, in a codon-dependent manner.

2.3. Elongation

The elongation step of protein synthesis involves two distinct events: A) the binding of the aminoacyl-tRNA to the acceptor site of the ribosome, followed by the formation of the peptide bond in the ribosomal peptide site; and B) the translocation of the ribosomal complex to a new codon. These events are catalyzed by two elongation factors (EF) in higher organisms; EF-1 and EF-2. Interestingly, these two EFs were the first molecules implicated in protein synthesis to be identified. EF-1 is a high molecular weight complex composed of three subunits, EF-1α (50 kDa), EF-1β (37 kDa) and EF-1γ (51 kDa) (Nombela et al., 1976; Slobin and Moller, 1975, 1976; Maessen et al., 1986, 1987; Lauer

et al., 1984; von der Kammer et al., 1991),. A fourth subunit, referred as EF-1δ, has also been identified (Carvalho et al., 1984) although the exact function for this peptide is still debated. This complex is involved in the GTP-dependent transport of aminoacyl-tRNA to the acceptor site of the elongating ribosome. The other elongation factor, EF-2, is a single polypeptide of 95 kDa that promotes the GTP-dependent shifting of the ribosome along the mRNA during elongation (Hershey, 1991; Merrick, 1992). Both EF-1 and EF-2 are highly studied complexes with intriguing regulatory mechanisms.

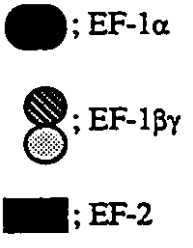
A) Binding of the aminoacyl-tRNA to the ribosome

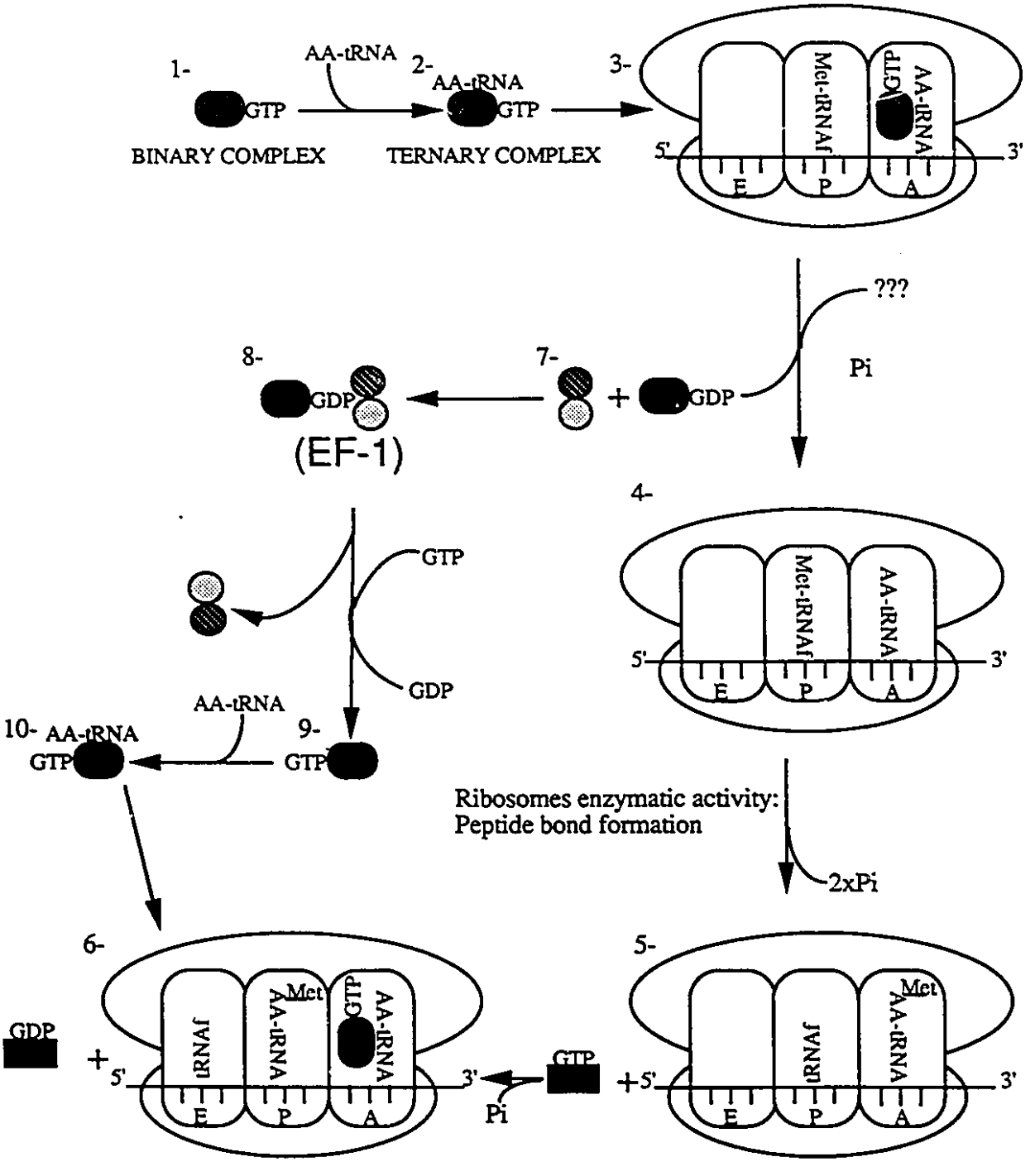
The first step in peptide elongation consists of the cyclic addition of aminoacyl-tRNAs to the acceptor site of the 80S ribosomal complex (Fig. 3). This is the role of the EF-1α subunit of the EF-1 complex. EF-1α is a 50 kDa molecule that binds GTP and aminoacyl-tRNA to form the EF-1α-GTP-aminoacyl-tRNA ternary complex (Jurnak, 1985; LaCour et al., 1985; Lauer et al., 1984; van Hemer et al., 1984; Kohno et al., 1986). Its principal function is to deliver the charged tRNA to the A site of the ribosome, in a codon-specific manner. After the correct match has been made, an unknown signal triggers GTP hydrolysis, and EF-1α is released from the ribosomal complex linked to GDP, in a binary complex. Formation of the peptide bond is catalyzed by the peptidyltransferase center of the large subunit of the ribosome (Nygard and Nilssen, 1990). It is the task of two polypeptides, EF-1β and γ, to facilitate the guanyl nucleotide exchange on EF-1α (Riis et al., 1990; Ryazanov et al., 1991; Janssen and Moller, 1988; Iwasaki et al., 1976) permitting the formation of a new ternary complex EF-1α-GTP-aminoacyl-tRNA, althought it has been reported that the γ subunit is sufficient for this activity (Iwasaki et al., 1976; Slobin and Moller, 1976).

The three EF-1 subunits undergo post-translational modifications. This aspect of EF-1α will be treated in later sections. As for EF-1β and γ, both peptides are

Figure 3. Schematic representation of eucaryotic translation elongation apparatus. EF-1α binds GTP (1) and aminoacyl-tRNA (2) to form a ternary complex that is translocated to the A site of the ribosome (3). A still unknown protein triggers GTP hydrolysis, and EF-1α is released from the ribosomal complex linked to GDP in a binary complex. The peptide bond formation between the two amino acids present in the A and P sites respectively is catalyzed by the peptidyltransferase center of the large subunit of the ribosome (4). Ribosome translocation is catalyzed by EF-2 in the presence of GTP hydrolysis (5). This translocation promotes the liberation of the A site for the delivery of another aminoacyl-tRNA by the EF-1α ternary complex (6, also 3) that is produced by the nucleotide exchange on the binary complex catalyzed by EF-1βγ (7, 8, 9), followed by the binding of a new aminoacyl-tRNA (10). This cycle will terminate only when a stop codon is positioned in the A site of the ribosome. This diagram is adapted from Merrick (1992), and Nygard and Nilsson (1990), with minor modifications.

Figure legend:




1-
AA-tRNA
2-
AA-tRNA
GTP
GTP
BINARY COMPLEX
TERNARY COMPLEX
3-
Met-tRNAf
AA-tRNA
GTP
5'
3'
E
P
A
???
Pi
8-
7-
GDP
GDP
(EF-1)
4-
Met-tRNAf
AA-tRNA
5'
3'
E
P
A
GTP
GDP
AA-tRNA
10- AA-tRNA
9-
GTP
GTP
Ribosomes enzymatic activity:
Peptide bond formation
2xPi
6-
5-
GDP
tRNAf
Met
AA-tRNA
GTP
AA-tRNA
5'
3'
E
P
A
Pi
GTP
tRNAf
Met
AA-tRNA
5'
3'
E
P
A

phosphorylated in Artemia, wheat germ, and rabbit reticulocytes (Janssen et al., 1988; Ejiri and Honda, 1985; Palen et al., 1990; Dholakia and Wahba, 1988). However, the precise consequences of such phosphorylation are still unclear, as both activation and repression of EF-1 activity have been reported in vitro. Another, more characterized modification of EF-1 is the phosphorylation at residue Ser89 of EF-1γ by the protein kinase p34cdc2 (Fig. 4A). This post-translational modification is believed to reduce the nucleotide exchange activity of the EF-1$\beta\gamma$ complex, presumably reducing the amounts of EF-1α ternary complex (Belle et al., 1989). p34cdc2 is a protein kinase that plays a crucial role in the control of the cell cycle. This relationship suggests that EF-1 activity is controlled in cell cycle-dependent fashion. Yet, the precise purposes for such a cell cycle control of protein elongation are still unclear.

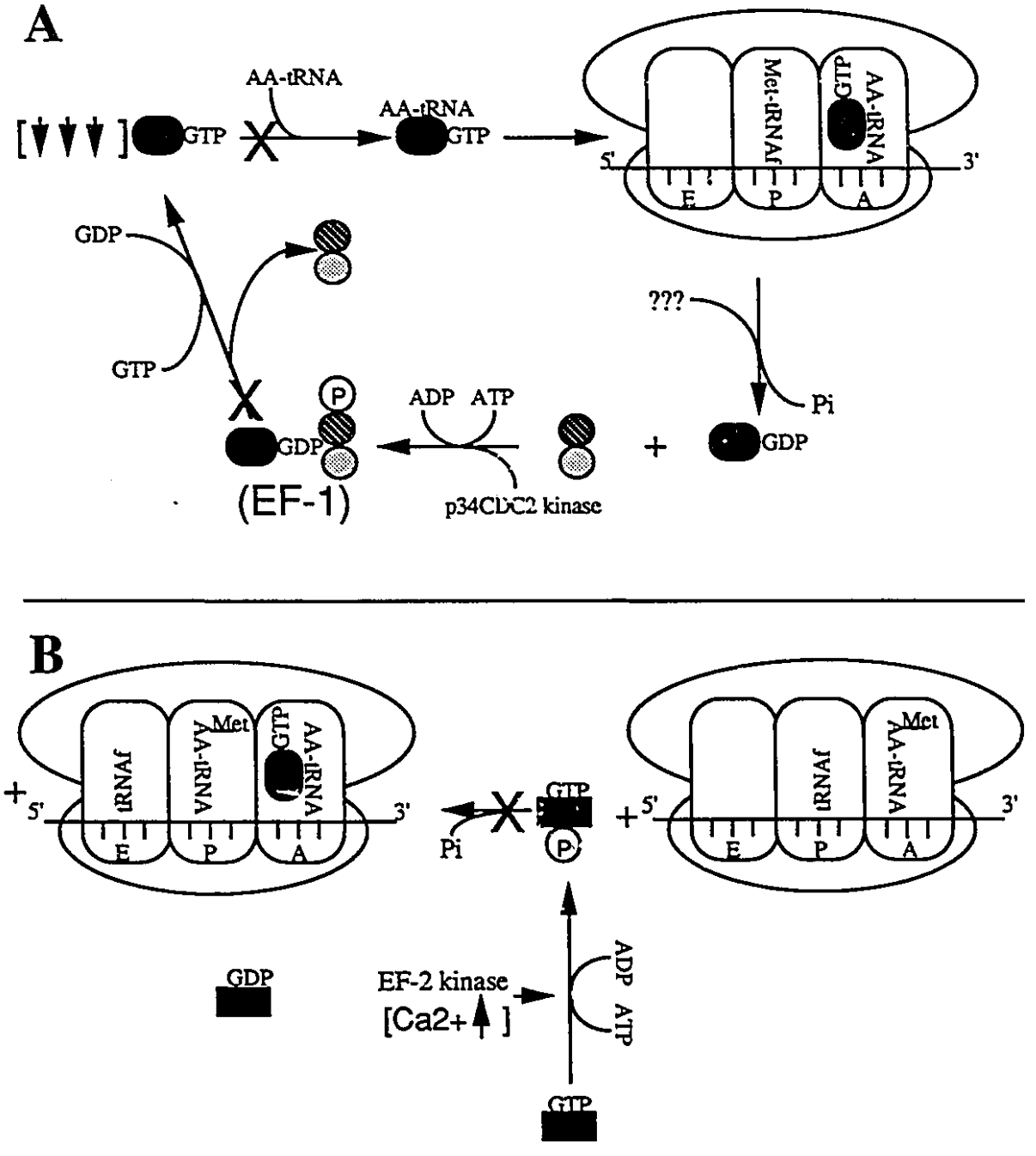
B) Translocation of the ribosome

After the formation of the peptidyl bond, the ribosome shifts one codon downstream, liberating the acceptor site for another EF-1α-GTP-aminoacyl-tRNA complex (Fig. 3). The shifting of the ribosome is presumed to be performed by EF-2, a single polypeptide of 95 kDa with a GTP-dependent activity (Hershey, 1991; Merrick, 1992). The exact mechanism by which EF-2 translocates the mRNA on the surface of the ribosome is still obscure, althought the EF-2-GTP complex alone appears to be able of translocating the mRNA in the elongating ribosome (Hershey, 1991). The addition of aminoacyl-tRNA is ensued by the formation of the peptide bond and translocation of the mRNA within the active ribosome. The cycle will terminate only when a stop codon (usually TGA) is positioned in the A site of the ribosome.

There is one peculiar post-translational modification of EF-2, which involves the conversion of a specific histidine residue to diphthamibe (van Ness et al., 1978). However,

Figure 4. Schematic representation of regulation mechanisms at the elongation step of translation.

A) Regulation by phosphorylation of the formation of the EF-1 complex. B) Regulation by phosphorylation of the EF-2 complex. The description of the regulation mechanisms is included in the text. The figure legend is the same as figure 3.


A
AA-tRNA
GTP
AA-tRNA
GTP
Met-tRNAf
AA-tRNA
GTP
5'
3'
E
P
A
GDP
GTP
???
Pi
P
ADP ATP
GDP
GDP
(EF-1)
p34CDC2 kinase
B
tRNAf
Met
AA-tRNA
GTP
AA-tRNA
5'
3'
E
P
A
Pi
GTP
P
GDP
EF-2 kinase
[Ca2+]
ADP ATP
GTP

yeast mutants lacking the enzymes responsible for this modification are viable, suggesting that it is not essential for protein synthesis (Bodley and Veldman, 1990). Another post-translational modification is EF-2 phosphorylation by a specific Ca_2^+/ calmodulin-dependent protein kinase III (EF-2 kinase; see Fig. 4B) (Nairn and Palfrey, 1987; Nygard et al., 1991). This kinase phosphorylates threonine residues at the N-terminal part of the protein, hampering protein synthesis by preventing ribosome translocation (Peter et al., 1990a; Ryazanov and Davydoka, 1989). In light of the fact that the concentration of Ca_2^+ increases during mitosis, investigators have suggested that the phosphorylation of EF-2 by the Ca_2^+-dependent EF-2 kinase is the principal mechanism by which protein synthesis activity is reduced during cellular division. This hypothesis was strengthen when it was shown that EF-2 was more phosphorylated during mitosis (Ryazanov and Davydoka, 1989, Celis et al., 1990). Ca_2^+-mediated phosphorylation of EF-2 is emerging as an important pathway by which protein synthesis is controlled during the cell-cycle traverse.

2.4. Termination

Compared to initiation and elongation, the termination event of peptide synthesis is simple. Elongation of the nascent peptide is terminated when a ribosome encouters a stop translation codon (usually UGA) at the acceptor site. To date, only one factor, the release factor (RF), has been characterized in eucaryotic species. RF, which is functional as a dimer, requires GTP for its activity (Konecki et al., 1977; Tate and Casey, 1974). Cloning of the cDNA has revealed that RF shares high sequence similarity with tryptophanyl-tRNA synthetases, indicating that a specialized tRNA may be responsible for termination (Lee et al., 1990). Functionally, RF promotes cleavage of the completed peptidyl-tRNA, releasing the newly synthesized protein into the cytoplasm. So far, no post-translational modifications or regulation mechanisms for this molecule have been uncovered.

3. Historical review of EF-1α

3.1. Introduction

The first publications on EF-1 date back as early as 1963 (Arlinghaus et al., 1963); since then, a large number of reports have been published on the subject. One reason for EF-1α's early discovery is that it is a very abundant cytoplasmic protein, representing at least 1% of the total cellular soluble proteins. This feature has made EF-1α analysis somewhat easy, even with older biochemical techniques. This section is a survey of the abundant literature on the original work that led to the identification of EF-1α. The first part of this section will be a brief survey of the work that resulted in the identification of prokaryotic EF-Tu, instrumental in the discovery of EF-1α's role in peptide synthesis. It will be followed by a description of the principal experiments that instigated the discovery of EF-1α's role in protein synthesis. Recent work, dealing with certain novel functional aspects of EF-1α, will be discussed in the last part of this section.

3.2. EF-Tu

In the early '60's, most of the functional studies on factors implicated in peptide elongation were achieved in prokaryotic species. It was reported that a soluble factor in bacteria, named EF-T, was capable of binding GTP and aminoacyl-tRNA (Arlinghaus et al., 1963; Nishizuka and Lipmann, 1966; Weissbach et al., 1970; Cooper and Gordon, 1969). EF-T was composed of two subunits, EF-Tu and EF-Ts, readily dissociable from each other. EF-Tu (39-42 kDa) was the most abundant of the two subunits, and the first to be purified to homogeneity (Miller and Weissbach, 1970). EF-Tu's ability to bind both GTP and aminoacyl-tRNA was shown by Millipore filtration. The EF-Tu-GTP or EF-Tu-GDP complexes are retained by the filter, whereas the ternary complex EF-Tu-GTP-aminoacyl-tRNA is not (Cooper and Gordon, 1969; Gordon, 1968) thus providing a mean

by which both complexes could be isolated and assayed. EF-Tu's implication in protein synthesis was readily demonstrated by adding purified ternary complex (GTP-EF-Tu-aminoacyl-tRNA) to already initiated ribosomes (Lucas-Lenard and Haenni, 1968; Skoultchi et al., 1969). In this experiment, dipeptidyl tRNA was rapidly formed between the initiator aminoacyl-tRNA and the added aminoacyl-tRNA. It was observed that the GTP molecule present in the ternary complex was hydrolyzed to GDP, that accumulated stoichiometrically to the amount of aminoacyl-tRNA bound to the ribosome (Gordon, 1969; Ono et al., 1969). The isolation of the binary complex EF-Tu-GDP strongly suggested that GTP must be hydrolyzed to liberate the aminoacyl-tRNA onto the ribosome. These results led investigators to conclude that EF-Tu was responsible for carrying the aminoacyl-tRNA to the ribosomes, and that GTP hydrolysis served as the energy source for this reaction.

3.3. EF-1α

The initial studies on mammalian elongation factors were done by Schweet and co-workers (Arlinghaus et al., 1963). They isolated two factors from rabbit reticulocyte lysate, referred to as EF-1 and EF-2, which were required for polypeptide synthesis. In considering the published work in bacteria, it was suggested that eucaryotic EF-1 was the functional homologue of EF-T. Indeed, EF-1 was a high molecular weight aggregate composed of subunits that can bind both GTP and aminoacyl-tRNA, such as EF-Tu and EF-Ts. However, the identification of such characteristics was impeded because ternary complexes, such as those identified in bacteria, were not easily purified from mammalian sources. The next few paragraphs will review the principal experiments that led to the demonstration of EF-1α's capacity to both form a ternary complex, and function in peptide elongation.

A) Formation of the ternary complex

The demonstration of EF-1α's capacity to form a ternary complex was performed using the same technique as in prokaryotes. Purified EF-1 from calf brain, liver, and reticulocyte lysates was incubated in the presence of GTP and aminoacyl-tRNA and filtered through a Millipore nitrocellulose filter. As was observed in prokaryotic systems, the ternary complex was not retained on the filter, providing a simple demonstration of ternary complex formation (Moon and Weissbach, 1972; Prather et al., 1974; Legocki et al., 1974). Interestingly, incubation in the presence of GDP did not result in the formation of a ternary complex, indicating that GTP is necessary for EF-1 binding to aminoacyl-tRNA. Moreover, EF-1 dissociated into monomers when incubated in the presence of GTP and aminoacyl-tRNA (Bolloni et al., 1974; Prather et al., 1974). This aspect of EF-1 permitted the isolation and characterization of a stable subunit. This subunit (50 kDa) was eventually shown to posses the capacity to form a ternary complex even in the absence of the high molecular weight EF-1 (Bolloni et al., 1974, Moon et al., 1972; Tarrago et al., 1973). This led to the attractive suggestion that the monomer of EF-1, referred to as EF-1α, was the functional equivalent of bacterial EF-Tu. Yet this hypothesis had to be demonstrated experimentally, and the next step was to show roles for both EF-1 and EF-1α in protein synthesis.

B) Function in peptide elongation

The first evidence for EF-1 ternary complex interaction with ribosomes was reported in 1973 by Weissbach et al., 1973. In these experiments, [^{32}P]GTP and [^{14}C]Phe-tRNA were incubated in the presence of EF-1, and the ternary complex was isolated by the Millipore filtration procedure. Incubation of labeled EF-1-GTP-Phe-tRNA complex with ribosomes in the presence of poly(U) provided evidence for the transfer of Phe-tRNA from EF-1 to the ribosome, followed by GTP hydrolysis. Interestingly, no GTP hydrolysis

occurs without poly(U) RNA, even if EF-1 interacts with the ribosomes (Weissbach et al., 1973). EF-1 did bound more strongly to the P-site of the ribosome rather than the A site indicating that the complex is present during peptidyl bond formation (Weissbach et al., 1973) although this is widely debated today. The demonstration of EF-1's role in protein synthesis was followed by several reports indicating that the catalytic activity of EF-1 was accomplished by the EF-1α subunit. EF-1α was capable by itself of promoting Phe-tRNA binding to ribosomes in an in vitro assay, in the presence of poly(U) mRNA (Ibuki and Moldave, 1968; Nolan et al., 1975; Weissbach et al., 1973) . The binding of EF-1α to the ribosome promotes GTP hydrolysis and release of the EF-1α-GDP binary complex that accumulates in the assay mixture, indicating that another factor must be present in the EF-1 complex to permit the guanosine nucleotide exchange reaction to occur (Weissbach et al., 1973; Lin et al., 1969; Slobin and Moller, 1976). This factor was later identified as EF-1βγ, the functional counterpart of bacterial EF-Ts (Ejiri et al., 1977; Slobin and Moller, 1978; Grasmuk et al., 1978; Motoyoshi et al., 1977). Therefore, the hypothesis that EF-1 was similar to prokaryote EF-T had been proven right. EF-1 is present in vivo as a high molecular weight aggregate of EF-1αβγ. EF-1α is the subunit responsible for the binding of aminoacyl-tRNA to the elongating ribosome during protein synthesis, in a GTP-dependent manner. The guanosine nucleotide exchange on the EF-1α-GDP binary complex is performed by the EF-1βγ subunits, permitting EF-1α-GTP to bond to an aminoacyl-tRNA to form another ternary complex.

3.4. Other cellular roles

The role of EF-1α in protein synthesis is well established. However, recent reports have linked EF-1α with other cellular processes. These include being part of messenger ribonucleoprotein particle (mRNP) complex (Greenberg and Slobin, 1987), being part of the valyl-tRNA synthetase complex (Motorin et al., 1988), binding actin (Yang et al.,

1990), being associated with the endoplasmic reticulum (Hayashi et al., 1989) or the mitotic apparatus (Ohta et al., 1990), and being involved in ribosome association (Herrera et al., 1991). So far, it is not clear whether it is EF-1α or related cellular proteins that are involved in these interactions, and no functional role for EF-1α in these interactions has been reported. Because most of these observations are recent, there is a general skepticism about the specificity of some of these interactions, especially because of EF-1α's abundance and high pI of 11. Nonetheless, it is possible that EF-1α may act as a general signaling device to other cellular compartments on the current state of the protein synthesis machinery.

4. Cloning of EF-1α

4.1. Introduction

The isolation of EF-Tu and EF-1α, and the characterization of their respective roles in protein synthesis, has been a major step in our comprehension of the overall translation machinery. Still, to understand EF-Tu/EF-1α's cellular role, the next logical step was to obtain information on the protein amino acid content. The previous biochemical characterization of EF-1α and its bacterial homologue, EF-Tu, and because this protein is so abundantly expressed, rendered the purification of EF-1α protein relatively easy. Eucaryotic EF-1α's amino acid sequence was first reported in 1984. This work was done in Artemia salina, and led to the isolation of the first eucaryotic EF-1α cDNA (van Hemert et al., 1984). It became evident that the EF-1α protein was a highly conserved protein, when the sequence of Artemia EF-1α was compared to that of bacterial EF-Tu, previously isolated in 1980 (An and Friesen, 1980; Amons et al., 1983). This aspect of EF-Tu/EF-1α was even more emphasized in 1986, when the first mammalian EF-1α cDNA was cloned from a human fibroblast library (Brands et al., 1986). Today, more than 25 EF-1α cDNA sequences from 15 different species have been cloned. The predicted molecular weight of the EF-1α protein varies between 48 and 52 kDa, depending on the species. The high

sequence similarity agrees with the previous biochemical analysis which had shown that prokaryotic EF-Tu and eucaryotic EF-1α are functionally similar. This first part of this section is devoted to the cloning of EF-1α genes and cDNAs both in prokaryotes and in eucaryotes. It is followed by a review of the isolation of EF-1α related genes in eucaryotes. The section is concluded by an analysis of the functional domains and the post-translational modifications of the primary amino acid sequences of the EF-1α protein.

4.2. In prokaryotic species

E. coli contains two genes coding for EF-Tu, known as tufA and tufB, whose complete nucleotide and amino acid sequences were published in 1980 (An and Friesen, 1980; Jones et al., 1980). The two genes in E. coli (strain K12) differ only in 13 positions, whereas the primary structures of the two gene products are identical except for one conservative amino acid difference in the C-terminus region of the proteins (An and Friesen, 1980; Jones et al., 1980; Laursen et al., 1991). The predicted amino acid sequence from the genomic clone was totally similar to the full peptide sequence obtained by chemical protein sequencing techniques (Jones et al., 1980). Since then, the tuf genes encoding EF-Tu have been cloned and sequenced from a variety of prokaryotic species, including Thermococcus celeriphilius, Thermus thermophilius, Thermoplasma acidophilium, and from several species of Bacillus and Chlamydia (Auer et al., 1990; Ludwig et al., 1990; Tesch and Klink, 1990, Peter el al, 1990a, b).

The genome of prokaryotes, except for certain Bacillus and Chlamydia species, always contains two EF-Tu genes. The primary amino acid sequences of these species' EF-Tu are highly similar (about 96%). More recently, genes encoding proteins with high but more divergent sequence similarity with EF-Tu have been isolated. The exact function of these EF-Tu-like proteins is unknown. There is one case where an EF-Tu-like protein binds specifically with a particular charged tRNA. The selB gene of E. coli codes for the

614 amino acid SELB protein, with a corresponding molecular weight of 69 kDa, that shares extensive sequence similarity with EF-Tu. SELB has been shown to bind GTP and the selenocysteyl-tRNA, and to be essential for the insertion of this special amino acid into elongating proteins (Forchhammer et al., 1990). The cloning of the EF-Tu genes has led to the identification of several EF-Tu-like proteins involved in specific aspects of the translation machinery (Cervantes et al., 1989; March and Inouye, 1985). This would suggest that prokaryotic species have evolved alternate translation factors required for the synthesis of proteins implicated in specific biological processes.

4.3. In eucaryotic species

The first eucaryotic species cDNA corresponding to EF-1α was cloned in the brine shrimp Artemia salina (van Hemert et al., 1983, 1984). It was quickly followed by two papers reporting the isolation of the yeast Saccharomyces cerevisiae's EF-1α cDNA (Nagata et al., 1984; Cottrelle et al., 1985a). The general similarity of the amino acid sequences between these two organisms and prokaryotic species was strikingly high (90% of the amino acid sequence totally conserved), and was in agreement with previous biochemical analysis that indicated functional similarity between all EF-1αs. From thereon, cDNAs coding for EF-1α have been isolated from a multitude of eucaryotic organisms such as Candida albicans (Sundstrom et al., 1990), carrot (Kawahara et al., 1992), Stylonychia lemnae (Bierbaum et al., 1991), Disctostelium (Yang et al., 1990), Mucor racemous (Linz et al., 1986a), Onchocerca volvulus (Alarcon and Donelson, 1991), Apis mellifera (Walldorf and Hoffman, 1990), Euglena gracilis (Montandon and Stutz, 1990), Drosophila melanogaster (Hovemann et al., 1988), and Xenopus laevis (Krieg et al., 1989). Again here, the EF-1α amino acid similarity between these distant and diverge species was extraordinarily high, making EF-1α one of the most conserved protein in evolution.

EF-1α cDNAs have been isolated from several mammalian species. The human EF-1α cDNA was first isolated in 1986, followed by the cloning of its corresponding gene in 1989 (Brands et al., 1986; Uetsuki et al., 1989). cDNA sequences from mouse (Lu and Werner, 1989) , rat (Ann et al., 1992; Shirasawa et al., 1992), and rabbit (Cavallius and Merrick, 1992) EF-1α have been reported in the last 5 years. The predicted amino acid sequence from the cDNAs has indicated that the EF-1α protein is extremely conserved in all mammalian species (99 to 100% conservation). Furthermore, in comparison with human EF-1α, bacterial EF-Tu is about 70% conserved, whereas yeast and tomato EF-1α are about 80% conserved. Another interesting characteristic of the mammalian EF-1α cDNAs is the highly conserved 3' UTR. The nucleotide sequence in this region is even more conserved than in the coding region (approximately 87% versus 92% respectively). This high sequence similarity is not observed in the 5' UTR. The exact reasons for such a high conservation of the 3' UTR are still not known.

4.4. EF-1α related genes

One interesting aspect of the EF-1α gene is that it is present in more then one copy per species. The presence of multiple active EF-1α genes has been shown in prokaryotes (An and Friesen, 1980), in different yeast species (Schirmaier and Philippsen, 1984; Sundstrom et al., 1990), in the fungus Mucor racemous (Linz et al., 1986b), in Artemia (Lenstra et al., 1986), in Drosophila (Hovemann et al., 1988), and in Xenopus (Dje et al., 1990). This is also the case in rat, where we have isolated both rat EF-1α and the highly homologous S1 (Ann et al., 1992). In yeast, both EF-1α genes can complement each other; only one EF-1α gene is necessary for normal metabolic activity (Cotrelle et al., 1985b). This is also truth in bacteria, where the expression of only one EF-Tu gene is necessary for survival (Hughes, 1990). The amphibian Xenopus laevis contains three active EF-1α genes (Dje et al., 1990). Two of them, EF-1S and 42Sp50, are only 63% similar, but both can

perform peptide elongation in an in vitro system (Mattaj et al., 1987). The third EF-1α gene in Xenopus, EF-1αO, is 90% homologous to EF-1S. So far no studies have shown that EF-1αO is able to elongate peptides, althought it does bind GTP and tRNA in vitro (Viel et al., 1987; Bourne et al., 1991).

The exact number of active EF-1α genes is species-specific. Up to nine EF-1α genes have been identified in a plant species. In animals, the number of EF-1α genes varies between one to fourth. In mammalian species, and also in Xenopus, the number of functional genes is unknown, due to the complication of sorting out EF-1α active genes from inactive retropseudogenes (Madsen et al., 1990; Abdallah et al., 1991). Retropseudogenes are intronless genes thought to be DNA copies of reverse-transcribed mRNAs, inserted at random into chromosomal DNA. There are several cases of retropseudogenes for highly expressed housekeeping genes in mammalian species, which usually out-number the functional copies of the homologous genes. The presence of up to 40 EF-1α retropseudogenes has been shown in pig, mouse and man (Madsen et al., 1990). This very large number of retropseudogenes in part reflects the rather high abundance of EF-1α mRNA in cells. Work is still in progress to identify other active EF-1α genes in mammalian species.

4.5 Sequence analysis

The chemical characterization of the EF-1α protein, and the cloning of its corresponding cDNA in a variety of species, have revealed some functional aspects of this elongation factor. The next few paragraphs will provide a brief review of the characterization of the functional domains and the post-translational modification of the EF-1α polypeptide.

A) Functional domains

The comparison of the amino acid sequence of the different EF-1α's has revealed some interesting aspects. One is the presence of GTP-binding domains, known as G-domains in th EF-1α/Tu primary amino acid sequence (Jurnak, 1985; LaCour et al., 1985; Kohno et al., 1986). The striking sequence and structure similarity between the G-domains of ras p21 and members of the RAS family and EF-Tu/EF-1α was reported in 1985 (van Damme et al., 1992; Jurnak, 1985; Valencia et al., 1991a, b). So far, three independent G-domains, and one putative domain, have been characterized in the EF-Tu/EF-1α peptide (Dever et al., 1987). These domains are totally conserved from yeast to man, indicating a strong evolutionary pressure related to the proper functioning of GTPase activity in all EF-1αs (Valencia et al., 1991a, b).

Other domains are also very highly conserved, such as the putative tRNA and EF-1βγ binding domains. Work done in E. coli has shown the presence of key amino acids responsible for binding aminoacyl-tRNA (Moller et al., 1987; Kinzy et al., 1992; van Damme et al., 1992). The sequence at the vicinity of these residues is conserved throughout all EF-Tu/EF-1α protein sequences reported so far (van Noort et al., 1984, 1985). As prokaryotic EF-Tu can perform its function with eucaryotic tRNAs, these conserved sequence elements probably play a role in tRNA attachment (Metz-Boutigue et al., 1989). There are also several highly conserved domains between all eucaryotic EF-1α's. While the exact function of these domains is not known, they are probably involved in some aspect of EF-1α function such as binding to mRNA, to ribosomes, or to the nucleotide exchange factor EF-Ts/EF-1βγ (van Damme et al., 1992). Therefore, the structure of the EF-1α protein is in good agreement with the known biochemical characteristic of EF-1α, including the formation of the ternary structure.

B) Post-translational modification

As mentioned above, the EF-1α protein has been chemically sequenced from three different sources. This has brought about the identification of interesting and novel post-translational modifications of the primary amino acid residues. These include the possible modification of glutamic acid to glutamate and methylation of lysine at different positions (Dever et al., 1989; Toledo and Jerez, 1989; Merrick, 1992). While the exact function of the first two mentioned post-translational modifications is still unclear, the methylation of the lysine at position 56 has been shown to play a role in controlling EF-Tu activity in bacteria (Toledo and Jerez, 1989). Another lysine residue at position 79 is also methylated; contrary to the lysine at position 56, this modification has been shown to be present in all species (Merrick, 1992). In fact, 4 of the 5 lysines that are methylated are the same (positions 36, 55, 79, 318) between A. salina and rabbit. It was found in Mucor racemous that the increase of methylation correlated with the elevation of overall elongation rate in the yeast-to-hyphae and spore germination stages (Hiatt et al., 1982). In contrast, purified hypo- and hyper-methylated forms of EF-1α were equally active in an in vitro system (Sherman and Sypherd, 1989). A more complex modification has been identified both in mouse and rabbit, and consists of the addition of glycerylphosphorylethanolamine at position 301 (Dever et al., 1989; Whiteheart et al., 1989). This modification may be present at position 374 in Artemia, altought it is absent in yeast (Merrick, 1992). The exact function for this post-translational modification remains unknown.

As discussed earlier, phosphorylation plays an important role in controlling protein synthesis activity. It has been reported that EF-Tu is phosphorylated at position 382 (Lipmann et al., 1993). Venema et al. (1991a, b) have also reported that mammalian EF-1α is phosphorylated in vivo and in vitro by PMA and protein kinase C respectively. They concluded that this modification results in activation of poly(U)-dependent poly(Phe)

synthesis, althought they did not identify the site of phosphorylation. However, the alignment of the part of the sequence from all known EF-Tu and EF-1α has revealed that the identified position corresponding to 382-The in E. coli is completely conserved in all sequences. Interestingly, the flanking sequences surrounding the threonine residue are not conserved, indicating that the threonine is the only amino acid to be totally conserved in EF-Tu/EF-1α in this particular region of the protein. This has led authors to propose that phosphorylation of EF-Tu and EF-1α is a general phenomenon in all organisms. However, in vivo functions for such phosphorylation have not yet been uncovered.

5. EF-1α Activity During Development

5.1. Introduction

The cloning of EF-1α cDNAs has provided investigators with tools to study the molecular aspect of this protein. It instigated the discovery that organisms contain several copies of the EF-1α gene. These genes code essentially for the same protein in lower organisms, but for slightly divergent proteins in higher species. They are differentially expressed, in a tissue-specific manner, during development and in adulthood. The differential expression of the EF-1α genes implies that tissues either have different needs for protein synthesis activity, or are composed of distinct protein synthesis machinery. These presumptions have arisen from data gathered from several systems including yeast, plants, and mammals. To simplify this section, the different systems, and the main conclusions derived therefrom, will be treated separately. Table-1 summarizes the present knowledge of EF-1α in some of the different systems.

5.2. In Yeast

Yeast EF-1α is encoded by two unlinked genes, known as TEF-1 and TEF-2, that have been isolated in two species, Saccharomyces cerevisiae (Nagata et al., 1984;

Table 1

Comparison between members of the EF-1α gene family of different species

Results in bold were obtained by the author of this thesis

Species	Gene	Sequence similarity (%)		Differential Expression			Function
(# genes)	Names	3' UTR[1]	A.acids	Embryonic	Adult	Aging	
S. Cerevisiae (2)	TEF1	<25	100	No difference	No difference	N.D.[2]	In peptide elongation
	TEF2			No difference	No difference		In peptide elongation
M. racemous (3)	TEF1	<25	100	Methylation level	High expression	N.D.	N.D.
	TEF2			Same	High expression		
	TEF3			Same	Low expression		
D. melanogaster (2)	F1	<25	92	All tissues	All tissues.	Decrease	N.D.
	F2			Pupal stage only	No expression.	N.D.	
X. Laevis (3)	EF-1αO	<25	>63	Until neurulation	Germ cell lines-specific.	N.D.	In peptide elongation
	EF-1αS			After blastulation	All tissues		Binds GTP, a.a.tRNAs
	42Sp50			Until neurulation	No expression		In peptide elongation
Mouse (1)	EF-1α	N.D.	N.D.	N.D.	Fibroblasts	N.D.	N.D.
Human (1)	EF-1α	N.D.	N.D.	N.D.	Liver, fibroblasts	Fibroblasts	In peptide elongation
Rat (2)	**EF-1α**	**<25**	**93**	N.D.	N.D.	???	In peptide elongation
	S1						

[1] <25; no significant similarity.

[2] N.D.; not determined or does not apply

Cotrelle et al., 1985a; Schirmaier and Philippsen, 1984), and in the dimorphic yeast Candida albicans (Sundstrom et al., 1990). The sequences of the cloned inserts have indicated that both tef genes code for identical proteins, but differ in the 5' and 3' UTRs. Both genes are expressed with no qualitative or quantitative differences during either growth or germination (Sundstrom et al., 1991). Surprisingly, only one of TEF-1 or TEF-2 is necessary for normal growth, as shown by insertion inactivation of these genes in Saccharomyces cerevisiae (Cottrelle et al., 1985b). The same results were obtained in the prokaryote Salmonella typhimurium where only one tuf gene is sufficient for survival (Hughes, 1990). These results indicate that neither genes or gene products have a unique essential role, therefore suggesting functional redundancy. However, Song et al. (1987) have demonstrated that EF-1α gene dosage is directly involved in translation fidelity, suggesting that both genes are necessary to permit the accumulation of adequately synthesized proteins. These results agree with reports predicting enhanced translation accuracy owing to an increase in the cellular pool of EF-1α, as shown by computer simulation of protein translation (Pingoud et al., 1990).

5.3. In Mucor racemous

Three EF-1α genes, known as TEF-1, 2 and 3, have been isolated in the fungus Mucor racemous (Linz et al., 1986a, b). These genes are highly similar in the coding region, but differ in their flanking regulatory or untranslated regions. As in yeast, these three genes code for the same protein, except that TEF-2 and TEF-3 contain a lysine instead of the glutamate in TEF-1 at amino acid position 41. It was suggested that the difference in the peptide chain elongation rates observed during the three morphogenetic events in Mucor racemous: the germination of spores, the formation of spores, and the transition between yeast-to-hyphal forms, may be due to the differential expression of these three genes. This is not the case, since no significant change in the mRNA levels of these three genes has

been detected during the morphogenetic events, although the mRNAs of TEF-1, 2 and 3 do not accumulate to the same levels (Linz and Sypherd, 1987). It is now hypothesized that the modulation of EF-1α activity is a result of post-translational modification, since a concomitant increase in lysine methylation of EF-1α occurs during this period (Linz and Sypherd, 1987; Hiatt et al., 1982; Sherman and Sypherd, 1989; Fonzi et al., 1985). Still, this does not explain why Mucor racemous or yeast contain 3 and 2 totally similar EF-1α genes respectively. It is unlikely that the advantage of multiple genes could be a simple quantitative effect, considering that yeast can grow normally with either of its two EF-1α genes deleted. So far, no function has been attributed to the extra EF-1α copies, except for the possible effect of EF-1α gene dosage on translation fidelity.

5.4. In plants

EF-1α gene expression has been analyzed in several plant species. A strong signal, obtained by in situ hybridization in regions of tomato that correspond to highly dividing cells has been reported (Pokalsky et al., 1989). In carrot, EF-1α transcripts accumulate to high levels in the spherical region of globular embryos, where actively dividing cells are observed (Kawahara et al., 1992). In contrast, a lower signal is detected in the less metabolically active suspensor-like regions of the same embryos. The developmental regulation of EF-1α gene expression was also analyzed in transgenic tobacco (Ursin et al., 1991). A gene fusion construct, consisting of the 5' and 3' UTRs from a tomato genomic clone of the EF-1α gene and the β-glucuronidase coding region, was used to produce the transgenic tomato. The pattern of β-glucuronidase activity in this system indicated that EF-1α gene is regulated, with increased expression corresponding to regions of high protein synthesis activity, including meristems, rapidly growing tissues and developing gametophytes. In addition, studies have shown that EF-1α expression responds rapidly to changes in growth pattern induced by hormone treatment. The principal conclusion of these

studies is that EF-1α levels are increased to elevate the rate of protein synthesis in tissues with high metabolic activity.

5.5. In Drosophila melanogaster

Drosophila contains two actively transcribed EF-1α genes, F1 and F2 (Hovemann et al., 1988; Walldorf et al., 1985). Contrarily to bacteria and yeast, these two genes differ in their expression profile, and code for proteins that are not totally similar in their primary amino acid sequence (92% similarity). According to the expression profile, F1 is the housekeeping gene, coding for EF-1α that is abundantly expressed in all cells. F2 expression is stage-specific. and its mRNA can only be detected early during development, at the pupal stage. Hovemann et al. (1988) have suggested that F2 exerts a specific function by preferring its own aminoacyl-tRNA pool, thereby controlling a certain aspect of the elongation step during development. Another possibility is that F2 is expressed in tissue already expressing F1, to increase the pool of active EF-1α. This could lead to enhanced translation accuracy in F1-F2-positive cells, as shown in yeast (Song et al., 1987).

5.6. In Xenopus laevis

Xenopus laevis contains three actively transcribed EF-1α genes that code for highly similar but different proteins (Krieg et al., 1989; Dje et al., 1990). The somatic and oocyte specific forms of EF-1α (EF-1αS and EF-1αO respectively) are 90% identical in their amino acid residues, while the immature oocyte form of EF-1α (42Sp50) is only 63% similar to EF-1αS (Viel et al., 1987, 1991; Coppard et al., 1991; Deschamps et al., 1991). The Xenopus genome also contain several retropseudogenes that have originated from the ubiquitous EF-1αS mRNA (Abdallah et al., 1991). EF-1αS is the housekeeping EF-1α abundantly expressed in all cells. Its mRNA accumulates from the midblastula point during embryogenesis, and is present in all tissues in adults (Krieg et al., 1989). The other two

EF-1α genes (EF-1αO and 42Sp50) have a more restricted, stage- or tissue-specific, pattern of expression. EF-1αO is transiently expressed in early development until neurulation, but its mRNA can only be found in male and female germ cells in adults (Abdallah et al., 1991; Mattaj et al., 1987; Deschamps et al., 1991; Frydenberg et al., 1991; Morales et al., 1991). 42Sp50 is expressed only in immature oocytes, and its mRNA cannot be detected in any adult tissues (Coppard et al., 1991; Viel et al., 1987; Deschamps et al., 1991). Compared to other species, the functions of these three gene products are well characterized. The three EF-1α genes are known to participate directly in protein synthesis (42Sp50, EF-1αS), or at least possess the capacity to bind GTP and aminoacyl-tRNAs (EF-1αO). 42Sp50 protein is also implicated in the storage of 5S RNA, and binds the ubiquitous TFIIIA transcription factor in the cytoplasm of immature oocytes. Interestingly, the three genes are expressed in a mutually exclusive fashion. In situ hybridization has indicated that EF-1αS is not expressed in adult germ cells, where abundant EF-1αO transcript can be detected (Abdallah et al., 1991). No EF-1αS mRNA can be detected in early previtellogenic oocytes, where abundant 42Sp50 mRNA is present (Dje et al., 1990). In adults, 42Sp50 and EF-1αO mRNAs are not detected in EF-1αS-positive tissues (Dje et al., 1990). The expression profile of the three EF-1α proteins implies that cells do not share the exact same protein synthesis machinery, and suggests a novel way to regulate translation at the elongation step.

5.7. In mammals

EF-1α cDNAs have been isolated from several mammalian species such as man, mouse, rabbit, and rat; the latter is the only mammal known to contain two EF-1α genes, EF-1α and the highly similar S1 (Ann et al., 1991, 1992; Shirasawa et al., 1992). Surprisingly, there are relatively few reports treating on EF-1α activity during mammalian development. One small study has indicated that EF-1α mRNA accumulates to higher levels in rat muscle than in liver, and that transformed cells in culture contain more EF-1α mRNA

than normal tissues in vivo (Sanders et al., 1992). EF-1α mRNA also accumulates to higher levels in cancer cells in vivo (Grant et al., 1992). That transformed cells contain more EF-1α mRNA is not surprising, especially because the overexpression of EF-1α in mouse 3T3 cells renders these cells more susceptible to oncogenic transformation (Tatsuka et al., 1992). As in lower species, the EF-1α protein accounts for about 3 to 5% of the total soluble protein content in different mammalian cell types (Brechet et al., 1986). It is therefore not surprising that the human EF-1α chromosomal gene can stimulate in vitro transcription better than the adenovirus major late promoter, which is one of the stronger promoter in mammalian cells (Uetsuki et al., 1989). Slobin's laboratory has reported post-transcriptional and translational control of EF-1α mRNA and proteins (Slobin and Jordan, 1984; Rao and Slobin, 1987); they have shown that EF-1α mRNA is more stable in non-proliferating Friend erythroleukemia cells than in their proliferating counterparts. In an ensuing paper, the same authors have shown that EF-1α mRNA, isolated as a messenger ribonucleoprotein from growth-arrested murine erytholeukemia cells, is not translatable in an in vitro assay (Slobin and Rao, 1993). This is in contrast to most RNPs, which are readily translatable in the same assays. This inhibition is caused by a trans-acting factor that binds to EF-1α mRNA in vivo. This effect seems specific, because the addition of the crude extract containing the trans-acting factor to an in vitro translation system containing MEL cell mRNAs partially and selectively inhibits EF-1α mRNA translation. These results, along with the post-translational modification discussed in section 3, suggest that mammalian cells control EF-1α level and activity at different post-transcriptional, translational and post-translational levels.

6. EF-1α activity during aging

6.1. Introduction

A common feature of many types of eucaryotic species is a progressive decline of protein synthesis during the aging process (Rattan, 1991). Practically all the studies have shown that the elongation step of translation is the most impaired by age. Thus far, there are no reports of age-related changes in the expression or activity of EF-1βγ. However, there are instances where changes in EF-1α levels and activity have been correlated with the age-related decline in protein synthesis. Yet, the evidences in favor of EF-1α's implication in aging are not totally convincing, and there is ample debate on the actual role of EF-1α in cellular and organismic senescence. This section will review the few relevant reports on this relationship.

6.2. Cellular aging

There are only a few papers reporting changes in the expression of EF-1α during cellular aging, and all the relevant work has been produced in normal human fibroblasts. Cavallius et al. (1986) have reported a decrease of 45% in active EF-1α protein in late passage senescent human fibroblasts (MRC-5). These results concur with work by Cavallius et al. (1989), who have shown a decline in EF-1α mRNA in senescent MRC-5 fibroblasts. In contrast to those observations is a paper reporting the isolation of a highly expressed clone in senescent WI-38 fibroblast which is totally identical to that of human EF-1α (Giordano and Foster, 1989; Giordano et al., 1989). These authors have shown by Northern analysis, using the full-length pSEN cDNA as a probe, that a 2.2 kb transcript accumulates 50-fold during cellular senescence. Since no attempts were made to distinguish EF-1α mRNA from other similar mRNA such as S1, it is not clear whether EF-1α mRNA levels are changed during cellular aging. Moreover, no major differences in EF-1α protein

level have been observed in young, old, or transformed human cells, implying that EF-1α is not directly involved in the attainment of the cellular senescent state (Rattan et al., 1986). More precise studies will be necessary to exclude EF-1α as a cause for the observed protein synthesis decline during the cellular aging process.

6.3. Drosophila aging

The most impaired step of protein synthesis during Drosophila aging is the binding of aminoacyl-tRNA to the ribosome, which is of course the primary role of EF-1α (Webster and Webster, 1982). This observation was followed by three independent reports indicating a sharp decline in the rate of EF-1α polypeptide synthesis and in EF-1α mRNA levels, starting early in adult life (Webster and Webster, 1983, 1984; Webster, 1985). This phenomenon precedes by only a few days a decline in protein synthesis in this organism, implying a direct relationship between EF-1α activity and aging (Webster and Webster, 1983). If EF-1α is responsible for the decline in protein synthesis, causing Drosophila to age, then restoring EF-1α activity in flies should prevent the aging phenomenon. This was the rationale for producing transgenic Drosophila with an extra copy of the F1 gene, that codes for the adult form of EF-1α (Shepherd et al., 1989). The F1 cDNA was subcloned down-stream of the hsp70 promoter, which is expressed in all cells in Drosophila. Two EF-1α-expressing transgenic Drosophila strains had a longer life span than nontransgenic controls, although there were variations in lifetime within both strains. Interestingly, transgenic strains incubated at 25.0 °C lived shorter than strains incubated at 29.5 °C, a temperature at which more transcription from the hsp 70 promoter of the EF-1α insert occurs. These experiments demonstrate that increasing the amount of EF-1α in flies has an influence in the life-span of Drosophila, and suggest a direct role for EF-1α in the aging phenomenon. However, a complete molecular characterization of EF-1α will be necessary to understand the exact role of this protein during Drosophila aging.

6.4. Mammalian aging

Again here there are conflicting reports on EF-1α level and activity during mammalian aging. This controversy probably reflects the fact that no systematic and precise analysis relating EF-1α and organismic aging has been produced. An age-related decline in the amounts and activity of EF-1α has been reported in old mouse and rat liver and brain (Rattan et al., 1991, 1986). These results agree with the observed general decline of protein synthesis during aging in these species (Rattan et al., 1991; Castaneda et al., 1986; Roels-De Schrijver, 1985). A similar drop in EF-1α mRNA has also been reported, although the decline in EF-1α transcripts observed in rat brain is much less significant than those observed in Drosophila. In contrast, these changes have not been observed in calorie-restricted Fischer 344 rats, despite a drop in total protein synthesis activity (Rattan et al., 1991). These experiments would suggest that EF-1α is not directly responsible for the age-related decline in translation. While it would be reasonable to speculate that EF-1α has a role in Drosophila aging, a direct implication of EF-1α in mammalian aging is still debatable, and awaits more accurate experimental analysis.

7. Cloning of rat S1

7.1. Introduction

EF-1α is encoded by several genes in all but a few species. Rats have also two members of the EF-1α gene family, EF-1α and the highly homologous S1. However, the cloning of S1 was a fortuitous event; the original goal was to isolate statin cDNA. Statin is a protein that can only be detected in the nucleus of nonproliferating cells. It is now understood that the protein product of the S1 cDNA, pS1, and statin are two totally independent polypeptides, sharing only a common antigenic determinant. This section will describe events that led to the isolation of the S1 cDNA. The first part of the section

introduces the present knowledge on statin, and describes the rationale that led to the isolation of S1. It is followed by a review of what is known about S1, including a sequence analysis between rat S1 and mammalian EF-1α. This section also contains data from a manuscript reporting on the isolation of rat EF-1α, of which I am a co-author (Ann et al., 1992).

7.2. Statin

Dr. Wang's laboratory has mainly focused on the identification and characterization of factors that are functionally implicated in the control of cellular growth arrest, i.e., quiescence and senescence. One strategy to identify such factors is to prepare specific probes using monoclonal antibody technology. Mice were injected with the Triton-insoluble extract of an in vitro aged culture of human fibroblasts obtained from a 66-yr.-old donor. Two monoclonal antibodies were obtained, referred to as S-30 and S-44. These two antibodies recognize an antigen, named statin, that is specifically localized in the nuclei of nonproliferating fibroblasts, but not in their proliferating counterparts (Wang, 1985a, b). Western analysis, immunoprecipitation and two-dimension gel electrophoresis have revealed that statin is a 57 kDa protein with a pI of 5.5. Electron microscopy using immunogold labeling has shown that statin is principally localized in the nuclear envelope. The data obtained from human fibroblasts is also applicable to all mammalian cells in culture or in vivo (Fedoroff et al., 1990; Sester et al., 1990; Muggleton-Harris and Wang, 1989; Connolly et al., 1988). Finally, the presence of statin is a reversible event, as the antigen is rapidly lost in cells that are stimulated to reenter the cycle, either by the addition of growth factors or removal of growth-contact inhibition (Wang, 1987; Ching and Wang, 1990)

An important characteristic of statin has been recently reported (Lee et al., 1992): It was shown that p57 statin is phosphorylated at serine residues in cultured cells. Immunoprecipitation assays with the S-44 antibody have revealed that p57 complexed with

another protein with a molecular mass of 45 kDa. This later protein was subsequently shown to be a protein kinase that can phosphorylate molecules such as immunoglobulins, enolase and casein. Immunoprecipitation and kinase assays have also revealed that statin-p45 kDa complex and phosphorylating activity is restricted to nonproliferating cells, such as human and murine 3T3 fibroblasts. These results suggest that statin forms a complex with a novel kinase that may be implicated in controlling the nonproliferating status of mammalian cells.

7.3. Isolation of the S1 cDNA

A necessary step in characterizing statin's role in the control of cellular growth was to clone its corresponding cDNA. The antibodies were used as probes, because no information on statin's protein sequence was available. A rat brain λgt11 expression library was screened with the S-30 and S-44 antibodies. Two independent positive clones, S1 and S2, with corresponding inserts of 1.7 and 0.7 kb, were isolated. Clone S1 was chosen for further characterization, because of the larger size of the insert. The S1 in vitro translated protein product (49 kDa) was shown to retain the antigenic determinant recognized by the S-44 antibody. These results showed that both statin and pS1 are recognized by the same S-44 antibody, and made S1 a good candidate for subsequent characterization (Ann et al., 1991).

The nucleotide sequence analysis indicated that the S1 cDNA is composed of 1719 bp, with only one large open reading frame of 1389 nucleotides. The first ATG codon conforms closely to the Kojak consensus translation initiation sequence, and is followed by an open reading frame that codes for a putative 463 amino acid polypeptide with a predicted molecular weight of 50.6 kDa. The coding region is flanked by a 114 bp 5' UTR and 216 bp of 3' UTR. The 3' UTR is extremely GC-rich (89%) in the first 120 bp after the stop translation codon. It is followed by 101 bp of normal GC-content. The polyadenylation

site (AAUAAA) is missing on the S1 cDNA, suggesting that more 3' UTR is present on the S1 mRNA.

We compared pS1 and statin protein characteristics. Statin amino acid composition was obtained by microsequencing $p57^{statin}$ purified by S-44 affinity chromatography, whereas pS1 composition was deduced from the S1 cDNA open reading frame. The characteristics of these proteins are entirely different. Statin and pS1 differ in their amino acid composition and molecular weight; a very important difference in their pI is also observed. These results strongly suggest that the only relationship between pS1 and statin is a common antigenic determinant (Ann et al., 1991). This type of cloning artifact is trivial, as there are several instances where totally different proteins share common epitopes. Efforts are being made to clone the statin cDNA using degenerative oligonucleotides derived from the microsequencing data recently obtained.

7.4. S1 sequence similarity with mammalian EF-1α

The nucleotide and predicted amino acid sequences of S1 were compared to those previously reported, using a computer search program into the Genbank data bank. Results of the search have indicated that S1 nucleotide and amino acid sequences were highly similar to those of mouse and human EF-1α (Ann et al., 1991; Uetsuki et al., 1989; Lu and Werner, 1988; Brands et al., 1986). The predicted coding region of S1 is 89% similar to that of mouse EF-1α, whereas the predicted amino acid sequence is 92% similar to that of both mouse and human EF-1α's (Table 2). Interestingly, the 5' and 3' UTR of S1 is totally divergent from those of the EF-1α mRNA of both species. This fact alone suggests that S1 is not the rat homologue of mouse and human EF-1α. Two more arguments that stress this point are that rabbit, mouse and human EF-1α are 99% similar at the protein level, and that they also share a very homologous 3' UTR.

Table 2

Sequence comparaison between different species EF-1αs and rat S1, with human EF-1α

Species	Sequence similarity (%)			Putative functional domains		Modified residues[2]
	5' UTR	3' UTR	A. acids	G-binding	AA-tRNA	CH3, GPEA, PO_4^-
Mouse EF-1α	<25[1]	>90	99.8	Conserved	Conserved	All conserved
Rabbit EF-1α	<25	>90	100	Conserved	Conserved	All conserved
Rat EF-1α	<25	>90	99.2	Conserved	Conserved	All conserved
Rat S1	<25	<25	92.6	Conserved	Conserved	All conserved

[1] <25: no significant similarity

[2] Known post-translation modified residues

However, to directly demonstrate this hypothesis, we cloned a partial cDNA (REF3) coding for rat EF-1α (Ann et al., 1992). Shirasawa et al. (1992) have also reported the isolation of a full-length rat EF-1α cDNA. The comparison between rat S1, rat EF-1α and human EF-1α is presented as part of Table 2. The predicted amino acid sequence of REF3 is 99% similar to that of mammalian EF-1αs. More importantly, the 3' UTR of REF3 is highly similar to that of mammalian EF-1α, demonstrating that it encodes rat EF-1α. That rat S1 encodes for a protein that is only 92% similar to rat EF-1α, and differs in its UTRs, is strong evidence that this species contains another member of the EF-1α gene family.

7.5. S10 gene

The S1 gene, referred to as S10, has been cloned and characterized. S10 contains 8 exons that span 10.7 kb in the rat genome. The poly (A) site, missing on the S1 cDNA, is localized 32 bp further down-stream from the cloned sequence, and is followed by the usual GT-rich region. No TATA or CAAT box has been identified upstream of the start

transcription site that was mapped by primer extension. Nevertheless, the putative S10 promoter does contain some known regulatory elements, such as Sp-1, AP-2 and ERE binding sites. More interestingly, 13 "E" box elements (CANNGT), important in myogenic terminal differentiation, can be distinguished in the putative promoter. The S10 gene also contains several unusual repeat sequences within its 7 introns. The exact function for these DNA sequences in the modulation of S10 expression is still awaiting molecular characterization.

The rat S10 gene was compared to the human EF-1α gene previously characterized (Uetsuki et al., 1989). These genes are structurally similar. They have the same number of exons, although the length and sequence of their introns differ considerably. Moreover, the EF-1α gene shares no evident sequence homology upstream of the transcription site with S1. The EF-1α gene also contains a CAAT and a TATA box that are not found in the S10 gene. It is thought that both genes have a common origin but have evolved in different routes, and that the S1 mRNA is transcribed from a different gene than that of EF-1α, both genes being under different regulatory controls.

8. Specific research aim

The isolation of S1 constituted the first report of an EF-1α-like gene in mammalian species, and led to a totally new project in Dr. Wang's laboratory. However, very little information was available about this novel S1 gene, except for its nucleotide and predicted amino acid sequences, and that it is expressed in rat brain, where it was originally isolated. Little information was also available about EF-1α molecular characteristics in mammals, such as its expression pattern and cellular distribution. To initiate the molecular characterization of S1, two main avenues of investigaton were undertaken in Dr. Wang's laboratory; the characterization of S1's cellular function and expression profile, both in comparison to EF-1α. The principal aim of my thesis was to broaden our knowledge on the

second avenue of investigation. Therefore, this thesis as focused on the characterization of S1 and EF-1α expession profile in mammalian species. To attain this objective, I have: collaborated in the cloning of a partial rat EF-1α cDNA, as mentioned in section 7 of this chapter, and prepared specific probes to both rat S1 and EF-1α; studied the expression profile and the cellular localization of both genes during development and aging; and cloned mouse and human S1 cDNA . The results obtained are presented in the next four chapters.

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CHAPTER 2

The cloning of the EF-1α cDNA in rat indicated that S1 was not this species homologue of mouse and human EF-1α (Ann et al., 1992; see chapter 1). Our preliminary results also suggested that the two mRNAs did not accumulate to the same levels in rat brain. This prompted us to hypothesize that S1 and EF-1α are two genes under different transcriptional control.

The following study was designed to test our hypothesis. Three principal questions were asked: What is the tissue distribution of S1 and EF-1α mRNA in rat? Is S1 mRNA tissue distribution the same in another mammalian species? Is there an age-dependent change in S1 and EF-1α mRNA levels in rat? To answer these questions, we develop specific molecular probes that could distinguish between the two mRNAs without ambiguity. The publication presented in chapter 2 is as it appears in the literature (Lee et al., 1992 J. Biol. Chem., 267; 24064-24068). Fig. 6 was added as an annex to support our conclusions.

TISSUE SPECIFIC EXPRESSION IN MAMMALIAN BRAIN, HEART, AND MUSCLE OF S1, A MEMBER OF THE ELONGATION FACTOR-1α GENE FAMILY

Abstract

Elongation factor-1 alpha (EF-1α) is a ubiquitous protein that functions in peptide elongation during mRNA translation. We previously reported the isolation of a rat S1 protein that is antigenically related to statin, a nonproliferation-specific protein; this S1 gene exhibits a high degree of homology with EF-1α. We constructed specific riboprobes to the two genes, based on the difference in the 3' noncoding regions of S1 and EF-1α mRNAs. Northern analysis and RNase protection assays have revealed that S1 mRNA is present only in brain, heart, and muscle, while EF-1α mRNA has been detected in all tissues surveyed so far. The same tissue specificity has been observed in mouse, suggesting that S1 expression is conserved between these two mammalian species. S1 transcript was detected in late brain embryogenesis (day 20), but in lower amounts than in 3-month-adult brain. We show that the relative levels of both S1 and EF-1α transcripts and their respective tissue abundances remain unchanged during the aging process. The function of S1 is not yet known; but these results suggest that it may be involved in specific control mechanisms for protein synthesis in tissues where cells (i.e., neurons and myocytes) are permanently locked in a state of nonproliferation.

Introduction

Translational control plays an important part in the modulation of gene expression (reviewed in Ref. 1). One of the key components of the protein translational apparatus is the ubiquitous and highly conserved elongation factor-1 alpha (EF-1α), a major protein constituent of all cells. EF-1α is part of the elongation factor-1 complex, which includes EF-1β and EF-1γ and promotes GTP-dependent binding of aminoacyl-tRNAs to ribosomes during peptide elongation. A striking feature of the EF-1α cDNA sequences from several species that have been reported in the literature is the very high similarity of the amino acid sequences (2-10). Using human EF-1α as the standard for comparison, mammalian species share between 99 and 100% amino acid similarity, while non-mammalian species share between 75 and 95% amino acid similarity. This high degree of identity has been interpreted as being due to conservation of the functional domains which are important in translation.

Another interesting aspect of EF-1α is the presence of several copies of active EF-1α genes in the genome of different eukaryotic species, such as Drosophila, and Xenopus (6, 11-13). These EF-1α like genes diverge more within a species genome than between species. The divergence between the nucleotide sequences of EF-1α genes within a species is thought to prevent homologous recombination between the two loci, but it is possible that changes in the primary amino acid sequence have led to a change in cellular function. Drosophila has two copies (F1 and F2) of the EF-1α gene, which are expressed at different time during development (11); while F2 is transcribed only in the pupal stage, F1 is a housekeeping gene expressed in all cells during development and in adulthood. Three EF-1α genes have been characterized in Xenopus (42Sp50, EF-1αO and EF-1αS) (12); 42Sp50 is expressed exclusively in oocytes, while EF-1αO is active from fertilization until early onset of neurulation. As in Drosophila, Xenopus contains only one active EF-1α gene

from late embryogenesis throughout adulthood. Whether these various genes encode EF-1α proteins with the same or different functions, specific for particular stages of differentiation, is still unclear. An interesting hypothesis is that overexpression of EF-1α genes would increase translational efficiency and fidelity; this notion is supported by work in yeast, where correlation exists between EF-1α levels and translational fidelity (14).

Another experiment confirming the translation fidelity hypothesis is that overexpression of EF-1α increases significantly the life span of transgenic Drosophila (15); it is thought that the extra EF-1α enhances protein synthesis, that normally declines during Drosophila aging (reviewed in Ref. 16). The Drosophila experiments have led investigators to suggest that EF-1α may play a predominant role in cellular senescence and aging (16); in fact, the decline in protein synthesis during aging in Drosophila is correlated with a decline in EF-1α activity and mRNA levels (17). As protein synthesis decreases in rat with age, a similar decline in EF-1α activity and mRNA level has been postulated to occur in mammalian species in general, including a decrease in transcript level in human senescent fibroblasts (18-21). No systematic work on EF-1α mRNA levels during mammalian aging has been published to date.

We recently reported the isolation of a 1.7kb clone, S1, from a rat brain cDNA library using monoclonal antibodies to statin, a protein found only in nonproliferating cells (22, 23). The deduced amino acid sequence of the S1 cDNA shares high homology (>92%) with human EF-1α, confirming its identity as a member of the EF-1α/S1 multigene family. S1 differs from rat EF-1α, however, and is encoded by a different gene. The cloning of rat EF-1α and S1 cDNAs indicates the presence of at least two members of the EF-1α gene family in mammalian species. Here we report the tissue distribution of S1 and EF-1α mRNAs by Northern analysis and RNase protection assays. We show that S1 mRNA is present only in brain, heart and muscle, while EF-1α mRNA is expressed in all tissues; we

also demonstrate that S1 and EF-1α mRNA levels and their ratio are modulated during development in rat brain, but do not change significantly during aging. We suggest that S1 gene expression is tightly controlled, and found only in tissues composed of cells that are permanently locked in a state of nonproliferation. This is, to our knowledge, the first report of an EF-1α-like gene whose expression is tissue-specific in mammalian species.

Experimental Procedures

Materials. [^{32}P]UTP (3000 and 650 Ci/mmol) was purchased from ICN Biochemicals Canada (Montreal, Quebec); the pGEM plasmid and Riboprobe synthesis system is from Promega (Madison, WI). All restriction enzymes were purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ) exept BstNI, which was from Stratagene (LaJolla, CA). Hybond nylon filters were from Amersham Corp. (Arlington Heights, IL). Rats were from Harlan Sprague-Dawley (Indianapolis, IN); for the aging experiments, Fischer 344 rats were purchased from the aging rat colony of the National Institute on Aging of the National Institutes of Health.

Construction and purification of S1 and EF-1α gene specific probes. Rat EF-1α (REF3) and S1 cDNAs were subcloned into pGEM-3z Xba-1/Sma-1 or EcoRI sites and designated pEF-1α-1 and pS1-7, respectively (23). pEF-1α-1 was digested with Ssp-1 restriction enzyme in order to produce a 235 nucleotide cRNA, containing 175 nucleotides from the 3' non-coding region of rat EF-1α. pS1-7 was digested with BstN-1 restriction enzyme to produce cRNA of 107 nucleotides, containing 99 nucleotides of the 3' non-coding region of S1. cRNA synthesis was performed using the protocol of the manufacturer (Promega) in the presence of [^{32}P]UTP and T7 RNA polymerase, followed by exhaustive DNaseI digestion. For RNase protection assays, labeled transcripts were subsequently purified by polyacrylamide gel electrophoresis. Briefly, after electrophoresis on 6% urea/polyacrylamide gel, the probe was cut off the gel using the autoradiogram as a template. The gel slices were then incubated at 37°C for 2-4 hours in a solution containing 2 M ammonium acetate, 0.1% SDS, and 25 mg/ml of yeast total RNA. The RNA was recovered by ethanol precipitation. S1-5 cDNA is an EcoRI/HinfI restriction fragment of the S1 cDNA containing 163 bp of the 5' noncoding region of S1 mRNA. The S1-specific oligonucleotide is a 32-mer in the coding region of S1 mRNA, that does not hybridize to

EF-1α transcript. HT7-3' is a BstEII/PstI restriction fragment of human EF-1α cDNA, containing 295 bp of the 3' noncoding region of human EF-1α mRNA.

Total and Poly (A)+ RNA isolation. Total RNA was isolated from different rat and mouse tissues using the acidic phenol/guanidium thiocyanate procedure (24). Poly(A)$^+$ was prepared by running total RNA samples three times on oligo(dT) columns, as described (25). Quality and quantity of the RNA were evaluated by U.V. spectrophotometry and by denaturing agarose gel analysis.

Southern analysis. To verify probe specificity, S1, rat EF-1α and mouse EF-1α cDNAs were run on a 1% agarose gel and transferred to a nylon membrane. Prehybridization of the filters was performed for 4 hours in a solution containing 6x SSC (1x SSC = 0.15 M NaCl and 0.015 M sodium citrate), 5x Denhart's solution (1x Denhart= 0.02% (w/v) polyvinylpyrrolidone, bovine serum albumin, and Ficoll), 100 μg/ml yeast total RNA, and 10 μg/ml denatured salmon sperm DNA at 58°C. The [^{32}P]UTP-labeled cRNAs were used at a final concentration of 2-4x 10^5 cpm/ml. After removal of the probe, the final wash was performed in a solution containing 0.1x SSC, 0.1% SDS at 65°C. Membranes were autoradiographed at -90°C.

Northern analysis. Total and poly(A)$^+$ RNA was run on a 1.2% agarose gel containing 7% formaldehyde, and transferred overnight to a nylon membrane. Filters were processed for prehybridization and hybridization as described above, and then autoradiographed at -90°C.

RNase protection assays. Protection experiments were performed essentially as described (25). Briefly, total or poly(A)$^+$ RNA (10-100 μg) was hybridized to 2.5x 10^5 cpm of pEF-1α-1 or pS1-7 cRNA in a solution containing 80% formamide, 1 mM EDTA, 0.4 M NaCl and 40 mM PIPES for 16 hours at 45°C. The hybridization mixture was then

digested with both RNase A and T1 for 60 minutes at 30°C (when pS1-7 was used as probe), or RNase T1 alone (for pEF-1α-1 probe). After proteinase K digestion and ethanol precipitation, samples were run on a 6-8% urea/polyacrylamide gel. Autoradiograms of polyacrylamide gels were performed at -90°C. The ratio of S1/EF-1α signals was estimated by transmission densitometry of the autoradiograms. Signals were also quantified by excising and counting radioactivity in the appropriate gel slices by scintillation counting. Results were standardized to the number of UTP nucleotides in each probe (23 UTP for S1 and 56 UTP for EF-1α).

Results

We have already reported the complete nucleotide sequence and predicted amino acid sequence of S1, and shown that the latter shares 92.6% homology to human EF-1α (22). Fig. 1A is a schematic diagram indicating the regions of homology and heterogeneity between the two proteins (26). There is significant conservation of the major EF-1α functional domains, such as the GTP and tRNA binding sites. It is thus likely that S1, like EF-1α, is involved in protein synthesis.

Our preliminary work has led us to hypothesize that S1 and EF-1α genes are regulated in different ways. It has been reported that protein synthesis and EF-1α levels change according to different physiological conditions (for review, see Ref. 16); we wished to examine the tissue distribution of S1 and EF-1α mRNAs in the rat, to compare their expression in vivo. Toward this end, specific riboprobes (cRNA) were constructed to the 3' noncoding region of both rat EF-1α and S1 mRNAs (Fig. 1, B and C). Rat tissues were tested for S1 and EF-1α expression by Northern analysis of poly(A)$^+$ RNA. Blots hybridized with the S1 cRNA identified a 2.0-kb mRNA (Fig. 2A) in brain, heart and muscle; no signal was detected in liver, kidney, lung, spleen, or testis. The same mRNA samples were analysed with the EF-1α specific probe, and a 1.8-kb mRNA was detected in all the tissues examined. Hybridization of the EF-1α probe served as a measure of RNA quantity and quality. We failed to detect any S1 mRNA in the negative tissues, using up to 20 μg of poly(A)$^+$ RNA and long term (2 week) exposures of the autoradiograms (data not shown).

We repeated the analysis of total and poly(A)$^+$ RNA by RNase protection. Fig. 2B shows that similar results were obtained, and that the protected probe was detected only in rat brain, heart, and muscle; no signal was detected in rat liver using 100 μg of total RNA

Figure 1. Amino acid sequence comparison between S1 and EF-1α, and construction of two gene-specific cRNA probes.

A) Schematic diagram of the comparison between S1 and human EF-1α (22, 26). Arrows: regions of 2-3 amino acid divergence between S1 and human EF-1α primary sequences. Dark boxes: sequences highly conserved between all known EF-1α and bacterial EF-Tu proteins. Gray boxes: region of divergence between these two types of EF-1α. Open boxes: GTP-binding domains. *: putative tRNA binding domains. Notice that all sequence divergences (arrows) between S1 and human EF-1α are outside functional and conserved domains.

B) The cRNAs used for Northern analysis and RNase protection are diagrammed (see "Experimental Procedures"). black lines: region transcribed by the T7 RNA polymerase (T7). Gray and open boxes: regions of high and low similarity between rat EF-1α and S1, respectively. cRNAs contain only antisense sequences from the divergent 3' noncoding regions of both genes. X: Xba-1, S: Ssp-1, M: Sma-1, E: EcoRI, B: BstNI, restriction sites.

C) Probe specificity: membranes containing plasmid digests of 1) rat S1 cDNA (1.7kb), 2) mouse EF-1α cDNA (1.7kb), and 3) rat partial EF-1α cDNA (935 bp including the 3' non-coding region) were hybridized to either pS1-7 or pEF-1α-1 antisense cRNA synthesized from the T7 promotor in the presence of [^{32}P]UTP. S1 and EF-1α cRNAs hybridized only to their respective cDNAs. pEF-1α-1 cRNA also hybridized with mouse EF-1α, because the 3' noncoding regions are conserved between the two species.

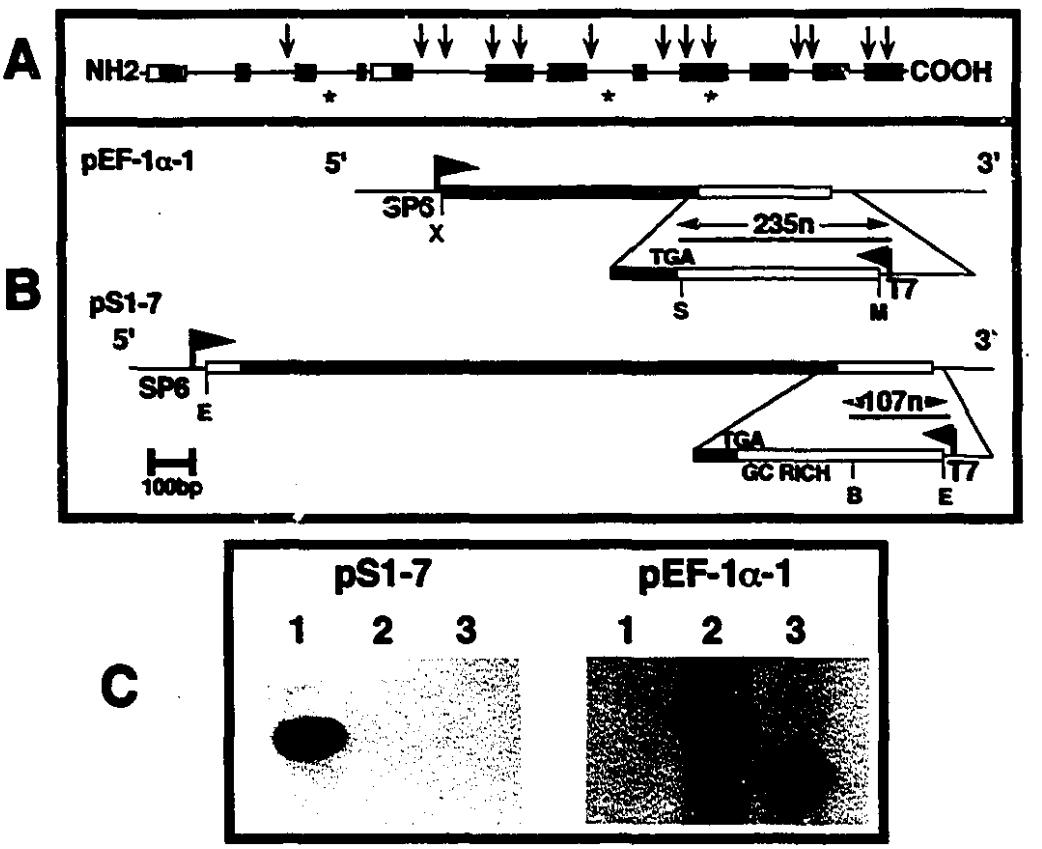
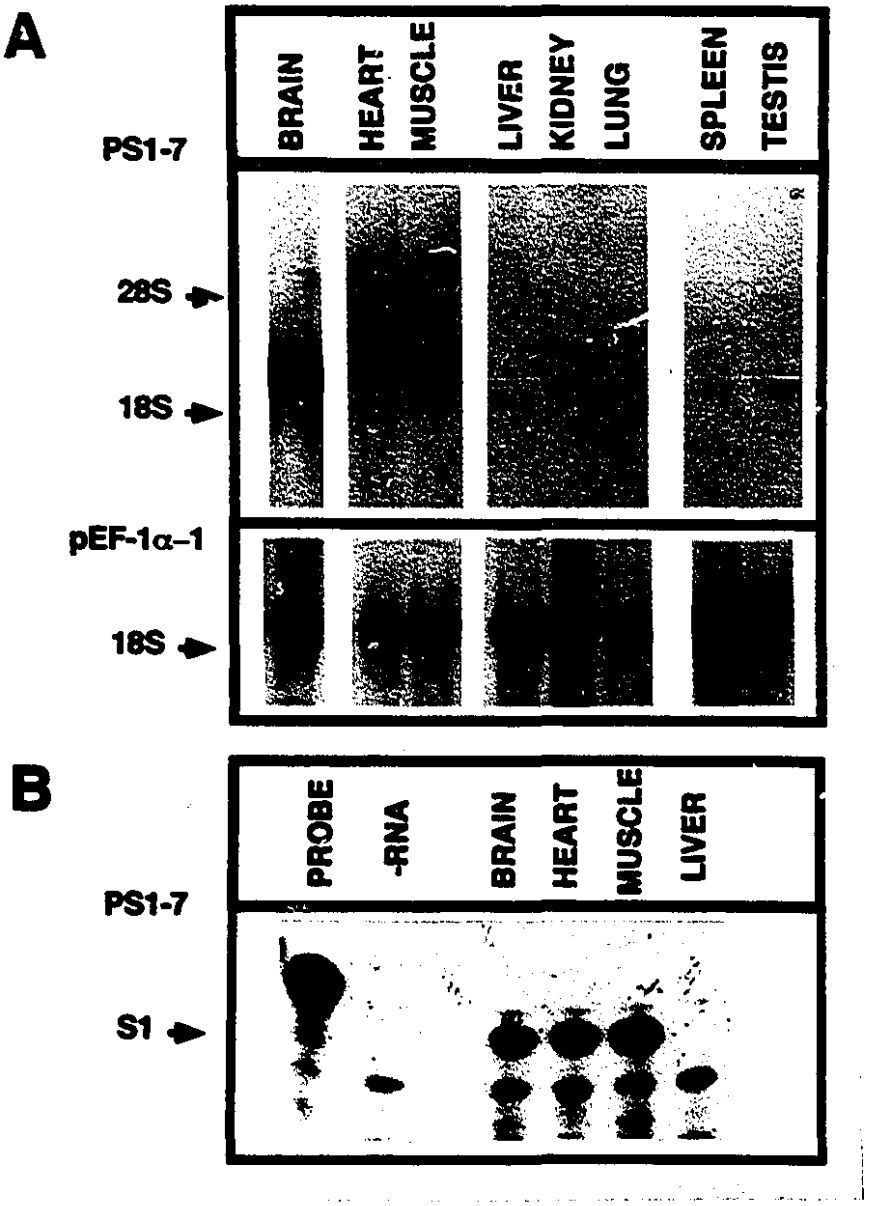

A
NH2
COOH
B
pEF-1α-1
5'
3'
SP6
X
235n
TGA
T7
S
M
pS1-7
5'
3'
SP6
E
107n
TGA
GC RICH
T7
B
E
100bp
C
pS1-7
pEF-1α-1
1 2 3
1 2 3

Figure-2. Expression of S1 and EF-1α mRNA in rat tissues.

A) Northern analysis. Poly $(A)^+$ RNA (5 μg) was separated on a 1.2% agarose gel containing formaldehyde, transferred to a nylon membrane, and hybridized with either pS1-7 or pEF-1α-1 [^{32}P]UTP labelled cRNA.

B) RNase protection assay. Rat total RNA (10 μg for brain, heart and muscle, 100 μg for liver) was hybridized with pS1-7 [^{32}P]UTP labeled cRNA, and RNase protection was carried out as described in "Experimental Procedures". Minus RNA (-RNA) indicates probe incubated without RNA. A nonspecific band, smaller than the protected fragment, is always observed even after polyacrylamide gel purification of probe and long incubation periods of the probe in RNase A and T1. Membranes and gel were autoradiographed at -90°C for A) pS1-7: 48 hrs; pEF-1α-1: 16 hrs; and B) 20 hrs.


A
BRAIN
HEART
MUSCLE
LIVER
KIDNEY
LUNG
SPLEEN
TESTIS
PS1-7
28S
18S
pEF-1α-1
18S
B
PROBE
-RNA
BRAIN
HEART
MUSCLE
LIVER
PS1-7
S1

(Fig. 2B), or 100 μg of poly(A)$^+$ RNA (data not shown). Other tissues such as kidney, lung, spleen, and testis were also negative (data not shown).

To eliminate the possibility that the lack of signal in S1-negative tissues was due to post-transcriptional modification of the 3' noncoding region of S1 pre-mRNA, filters containing rat brain and liver total RNA were hybridized with either a specific oligonucleotide to the coding region of S1, or a cDNA to the 5' noncoding region of S1 mRNA. Using these two probes, we still could not detect any signal in liver (Fig. 3). Other Northern analysis using 5' and 3' S1-specific oligonucleotides also failed to detect S1 transcripts in tissues other than brain, heart, and muscle (data not shown). The same RNA preparations were identified to contain the message for EF-1α when probed with a human EF-1α 3' noncoding region cDNA (Fig. 3)

To expand our observation of tissue-specific regulation of the S1 gene in rat, we analysed total and poly(A)$^+$ RNA from different mouse tissues by RNase protection and Northern analysis. Fig. 4 shows that the tissue-specific expression of S1 mRNA in brain, heart, and muscle is conserved between the two species. RNase protection was also performed in human muscle, where similar results were obtained (data not shown). These results taken together indicate that tissue-specific expression of the S1 gene is conserved in mammals (rat, mouse, and human); they also show that the 3' noncoding region of the S1 message is conserved among the three species.

There are several reports of the regulation of EF-1α genes during development and aging in non-mammalian species. To our knowledge, no systematic work has been attempted to demonstrate modulation of EF-1α gene expression during the normal mammalian lifespan. To answer this question, we monitored S1 and EF-1α gene expression during development and aging in rat; we analysed S1 and EF-1α mRNA levels and S1/EF-1α mRNA ratios by RNase protection assays, using S1- and EF-1α- specific

Figure-3. Detection of S1 mRNA in brain and liver using different S1-specific probes. Total RNA (10 μg from either tissue) was separated on a 1.2% agarose gel containing formaldehyde, and transferred to a nylon membrane. Probes used for hybridization are (see "Experimental procedures"): S1 oligo. coding, 32 mer oligonucleotide to S1 coding region; S1-5', 163-bp 5' noncoding region cDNA; HT7-3', 295-bp cDNA to the 3' noncoding region of human EF-1α. Filters were exposed for 24 hrs. Note that S1 is detected only in brain, while EF-1α can be observed in both tissues.

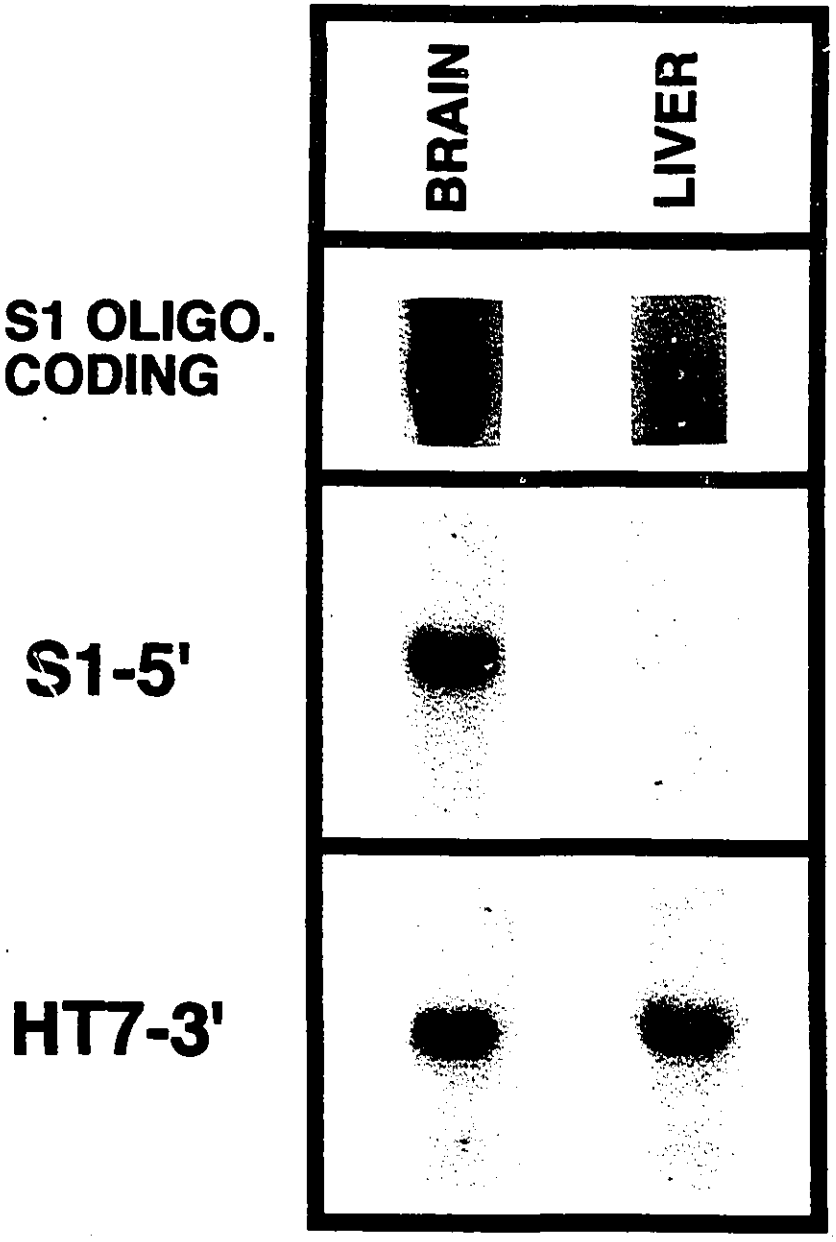
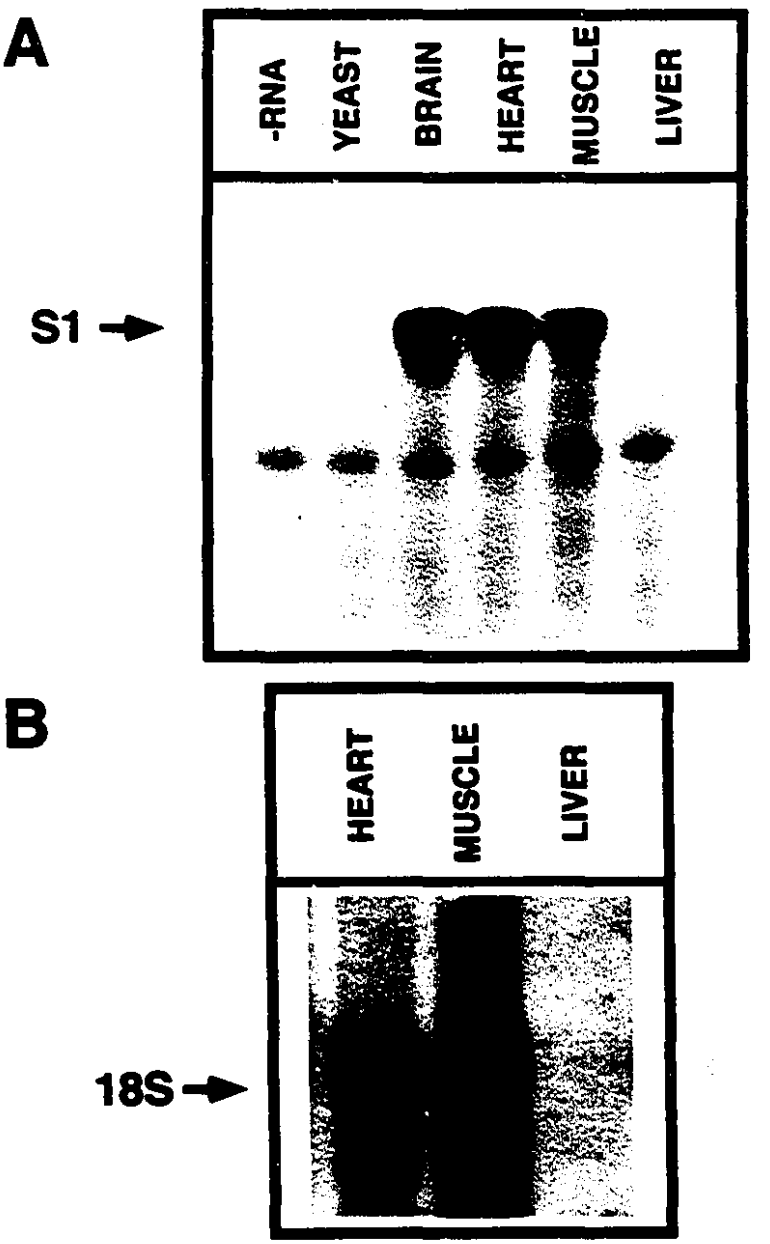

BRAIN
LIVER
S1 OLIGO.
CODING
S1-5'
HT7-3'

Figure 4. Expression of S1 in mouse tissues.

A) RNase protection. Total RNA (10 μg for all lanes except 100 μg for liver) was hybridized with pS1-7 [^{32}P]UTP labeled cRNA, and RNase protection was carried out as described under "Experimental Procedures". The gel was autoradiographed for 20 hrs.

B) Northern analysis. Poly(A)$^+$ RNA (5 μg) of heart, muscle and liver was separated on a 1.2% agarose gel containing formaldehyde, transferred to a nylon membrane, and hybridized with pS1-7 [^{32}P]UTP labeled cRNA. Membrane was autoradiographed for 50 hrs. Note that here also, S1 is sean in brain, heart, and muscle but absent in liver.


A
-RNA
YEAST
BRAIN
HEART
MUSCLE
LIVER
S1
B
HEART
MUSCLE
LIVER
18S

cRNAs in rat brain, heart, and liver of different ages. In all, three sets of rats between the ages of 3 and 26 months were studied, as well as brain from 20-day-old rat embryos. Fig. 5 shows representative experiments: we noticed a decrease in EF-1α mRNA levels from fetal to adult brain, but did not detect any significant changes in EF-1α transcript levels in brain during the adult life span of the rat. A similar constant level of EF-1α was also observed in rat heart and liver (3 to 26 months). As for S1 mRNA, we sometimes detected slightly lower amounts of S1 transcript in fetal and 3-month-old than 9- to 26-month-old brains, where S1 levels are constant (as shown in Fig. 5). We also did not observe any S1 transcript in old rat liver, indicating that the negative tissue-specific expression of S1 in liver is maintained during the aging process. We obtained similar results by Northern analysis of 3- to 26-month old brain and liver total RNA, hybridized with S1-specific oligonucleotide and HT7-3' probes (Fig. 6; annex).

To compare EF-1α and S1 ratios precisely in the different tissues, we measured signal intensity by transmission densitometry and/or scintillation counting of slices cut from the polyacrylamide gel (Table 1). Adult (9-26 months) heart had a higher S1/EF-1α ratio (1.62) than did adult brain (1.29). More significantly, we observed a 5-fold increase in the S1/EF-1α ratios from fetal (0.27) to adult (1.29) brain; the change in the ratios is primarily due to a decrease in EF-1α mRNA levels, and also, to a lesser extent, to an increase in S1 mRNA levels. These results suggest that there are temporal changes in S1 and EF-1α gene expression during development and early adult life, while the levels of both transcripts are constant during the aging process.

Figure 5. Analysis of S1 and EF-1α mRNA levels during rat aging by RNase protection. Total RNA (10 μg) of brain, heart, and liver of rats of different ages was hybridized with 2.5 x 10^5 cpm of pS1-7 and pEF-1α-1 cRNAs. We did not detect any significant increase of signal using a higher concentration of probe in previous experiments (data not shown). RNase protection was carried out as described in "Experimental Procedures". To reduce background, the hybridization mixture was digested with RNase T1 alone for 60 minutes at 30°C. Samples were run on an 8% urea/polyacrylamide gel, in order to separate the protected probes. Gels were autoradiographed at -90°C for 16 hours, except for brain (4 hours). E: embryonic days; M: months post natal.

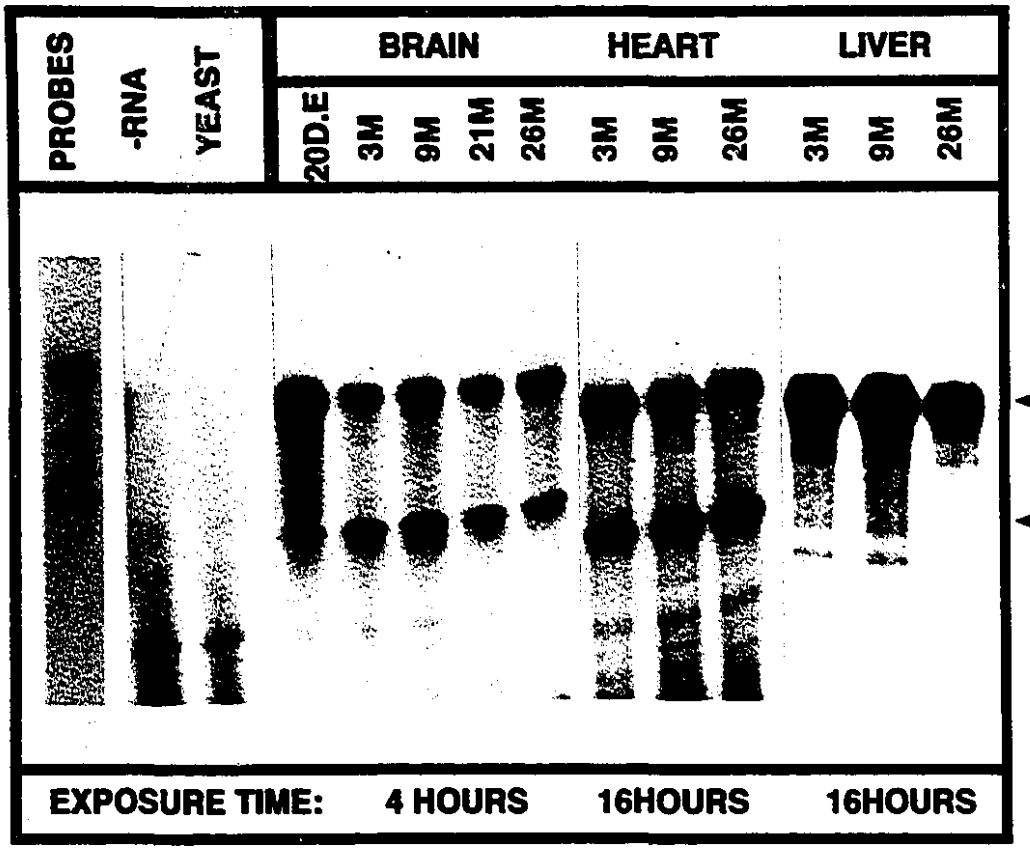

PROBES
-RNA
YEAST
BRAIN
HEART
LIVER
20D.E
3M
9M
21M
26M
3M
9M
26M
3M
9M
26M
EF-1α
S1
EXPOSURE TIME:
4 HOURS
16HOURS
16HOURS

Figure 6. (Annex). Analysis of S1 and EF-1α mRNA levels during rat aging by Northern blots.

Total RNA (5 μg) of brain and liver from rats of different ages was separated on a 1.2% agarose gel containing formaldehyde, transferred to a nylon membrane, and hybridized to either 2.5 x 10^5 cpm of S1 oligonucleotide coding, or HT7-3' cDNA. We did not detect any significant increase of signal using a higher concentration of probe in previous experiments (data not shown). Gels were autoradiographed at -90°C for 48 hours. M: months postnatal.

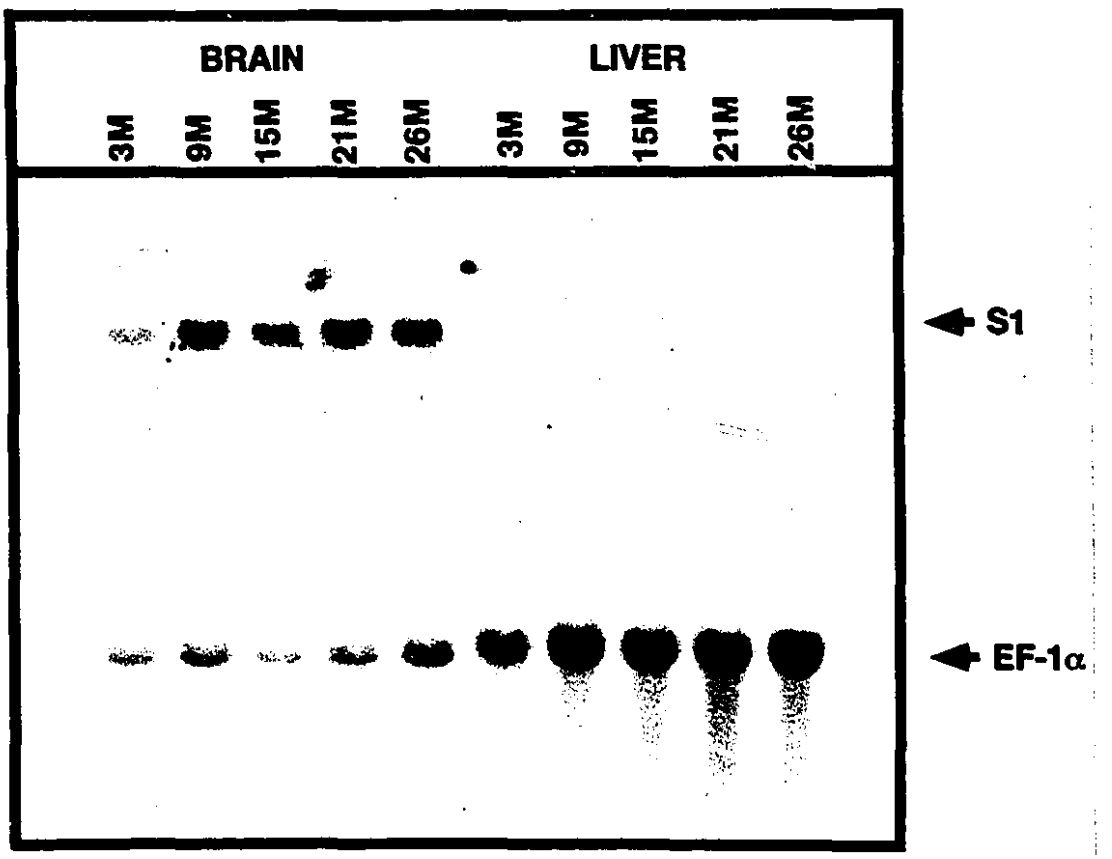

BRAIN
LIVER
3M
9M
15M
21M
26M
3M
9M
15M
21M
26M
S1
EF-1α

Table 1

S1/EF-1α ratio in developing and adult tissues

The results shown are means +/- S.E. of 3 to 6 independent experiments with duplicate determination.

Tissue	S1/EF-1α ratio
brain embryo[a,b]	0.27 +/- 0.05
brain adult [b,c]	1.29 +/- 0.03
heart adult [b,c]	1.62 +/- 0.09
liver adult [c,d]	>100

[a] Three brains of 20-day-old embryos were used.

[b] Ratio was calculated by transmission densitometry, as described in "Experimental Procedures."

[c] Six adults of 9 to 26 months were used.

[d] Ratio was calculated by liquid scintillation counting, as described under "Experimental Procedures."

Discussion

We report here the tissue-specific expression of an EF-1α gene analogue, S1, in rat and mouse brain, heart, and muscle. By vitue of its high amino acid sequence similarity to EF-1α, we suggest that pS1 may perform a similar function in peptide chain elongation in these three tissues. Although S1 cDNA was isolated with antistatin antibodies, primary statin amino acid sequence analysis reveals that S1 and statin are two unrelated proteins which share only an antigenic determinant (Lee, M.-J. and Wang, E. unpublished results). We could not detect S1 mRNA in any other tissues, by either RNase protection or Northern analysis of total and poly(A)$^+$ (100 μg) RNA; statin, in contrast, is expressed in all tissues, including liver. We also did not observe any signal of other sizes in negative tissues by Northern analysis with probes spanning the S1 message, excluding the possibility of the absence of signal due to alternative splicing of the 3' non-coding end of the S1 pre-mRNA. Therefore, our results suggest that S1 is a second member of the EF-1α gene family, whose expression is regulated at the transcriptional or post-transcriptional level in adult mammalian tissues. Although modulation of the expression of multiple EF-1α genes during development has been shown in lower species such as Drosophila and Xenopus, to our knowledge this is the first demonstration of a tissue-specific expression of an EF-1α-like gene in adult tissues.

In contrast to Xenopus and Drosophila, where variation of EF-1α gene expression is observed only during early development (before neurulation) (6, 11-13), we detected S1 mRNA in 20-day-old rat embryo brains; we also detected large amounts of EF-1α mRNA in fetal, compared to adult, rat brains. In fact, the S1/EF-1α ratio, as measured by densitometry, is significantly lower in fetal than in adult rat brain (9-26 months). This is in large proportion due to a decrease in the amount of EF-1α mRNA during late embryogenesis and early adult life (20-day embryo to 3-month adult), although we also observe a slight

increase in the S1 mRNA levels during the same time frame. These results indicate that S1 and EF-1α gene expression are regulated differently during development. We are presently analysing more precisely S1 and EF-1α mRNA levels during development and early onset of life, to verify which tissues and cell types express one or both genes.

Numerous reports relate a decrease in protein synthesis with cellular and organismic aging (for review, see Ref. 16). In Drosophila, the step in protein synthesis most impaired by age appears to be peptide elongation. Since EF-1α activity and its mRNA levels dramatically decrease during aging in Drosophila, it has been suggested that EF-1α may be a main regulator of the aging process, at least in this organism. Because it has been reported that protein synthesis decreases in aging rat liver and brain, we wanted to investigate whether the levels of S1 and EF-1α, and their ratio, change during the rat life span; we have shown by RNase protection and Northern analysis that there is no significant change in EF-1α mRNA levels in brain, heart, and liver during the adult rat life span (3-26 months). The slight differences observed are probably due to variations expected when dealing with individual animals. As for S1 mRNA, we sometimes detect a small increase in early adult life (20-day fetal brain to 9 months), but S1 mRNA levels do not change significantly from 9 to 26 months. We also did not detect any S1 mRNA in old liver, suggesting that the tissue-specific expression of S1 is maintained during aging.

Although brain, muscle, and heart tissues differ in their morphogenesis and function, they share one common property: a significant portion of the cells (myocytes and neurons) become fully differentiated and permanently locked in a state of nonproliferation shortly after birth. Preliminary results using cell culture indicate that S1 expression is limited to myogenic cell lines (data not shown). Therefore, our results suggest that S1 expression is limited to cells that, once totally differentiated, are incapable of re-entering the cell cycle. Work in yeast suggests that increased expression of EF-1α elevates translational

fidelity (14). We are presently investigating S1 gene expression and protein function during myogenic differentiation, and we will try to correlate S1 expression to the maintenance of terminal differentiation status and translational fidelity.

The many reports of EF-1α genes, such as those related to actin (27), mitotic spindle (28), and heterogeneous nuclear ribonucleoproteins (29), prompt us to consider that, alternatively, the S1 gene product may function in a totally different fashion than EF-1α; we are investigating this possibility by experiments such as evaluating the in vivo function of the S1 protein via sense and antisense transfection of different cell types. Future results will reveal why S1 is only expressed in brain, heart and muscle.

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CHAPTER 3

We showed in chapter 2 that S1 mRNA is detectable only in brain, heart, and muscle whereas EF-1α mRNA can be detected in all tissues studied so far. These results led us to hypothesize that S1 gene expression was tissue-specific, and that the S1 transcript can only be found in the three mentioned tissues. However, it is possible that certain cell types are S1-positive in liver, and that no signal was detected due to the rarity of the message in RNA samples in liver homogenate. Whereas our previous studies were based on analysis of RNA samples from various tissue homogenates, this chapter describes in situ hybridization experiments, showing the tissue distribution of the two mRNAs in liver and brain sections.

The principal objective of the following studies is to confirm the results presented in chapter 2. To do so, we developed a method to verify the incorporation of digoxygenin-11-UTP during in vitro transcription of the specific cRNAs described in chapter 2. We then performed in situ hybridization on frozen sections of rat brain and liver. The publication presented in chapter 3 is as it appears in the literature (Lee et al., **1993** J. Histochem. Cytochem., 41; 1093-1098). An extra panel was added as an annex to Fig. 2 to support our conclusions.

LOCALIZATION OF S1 AND ELONGATION FACTOR-1 α mRNA IN RAT BRAIN AND LIVER BY NON-RADIOACTIVE IN SITU HYBRIDIZATION

Abstract

Elongation factor-1 alpha (EF-1α) is an ubiquitous, highly conserved protein that functions in peptide elongation during mRNA translation. We recently reported that, as do lower species, mammals also contain a second EF-1α-like gene (S1). Unlike EF-1α, which is present in all tissues, S1 mRNA is detected only in brain, heart, and muscle by Northern analysis and RNase protection assays. In this report we present the identification of S1 and EF-1α messages by non-radioactive in situ hybridization in brain and liver. We show that, using this technique, we can detect S1 mRNA only in brain and not in liver. On the other hand, EF-1α mRNA is present in all cell types that we have studied so far. We could not detect any S1-positive cells in liver. The similar pattern of expression between S1 and EF-1α in brain hippocampus may suggests that in certain cases, both genes are expressed in the same cells. The results presented here are in agreement with our previous finding, that mammalian species contain a tissue-specific EF-1α-like gene, S1. The presence of a second EF-1α-like transcript within fully differentiated cells suggests a novel cell type-specific gene expression, whose function may be related to protein synthesis in permanent growth-arrested cells in brain, heart, and muscle.

Introduction

It is becoming increasingly evident that protein translation plays an important role in the control of gene expression (see review in reference 8). One of the key components of the protein translational apparatus is elongation factor-1 alpha (EF-1α), a 50 kDa protein that promotes GTP-dependent binding of aminoacyl-tRNA to the ribosomal complex during peptide elongation. It is a very highly conserved protein, present in high abundance in every cell of all organisms that have been studied so far (4, 7, 9, 10, 12, 13, 20, 22, 23).

The genomes of different eucaryotic species have been shown to contain several active EF-1α genes (3, 9, 24). In Drosophila (9) and Xenopus (5), the expression of the different EF-1α genes is developmently regulated; although all genes are active during development, only one EF-1α gene is expressed in adult tissues. Thus far, no function for the extra EF-1α protein has been discovered, but it has been suggested that the overexpression of a stage-specific form of EF-1α may result in increased translational accuracy, as observed in yeast (18).

Mammalian species also contain a second EF-1α-like gene. We recently reported the cloning of a 1.7kb cDNA, S1, that shares high sequence similarity with the known human EF-1α (>92% amino acid) (1, 2). We showed by Northern analysis and RNase protection assay that the S1 gene is expressed in adults, but only in three well-defined tissues, brain, heart, and muscle, whereas we detected EF-1α transcript in all tissues (11). To verify the tissue specificity of S1 gene expression, we show in this report the tissue distribution of S1 and EF-1α mRNA in brain and liver by in situ hybridization. We also present a method to verify the production of full length, digoxigenin-labeled cRNA, and its application in localizing messenger RNA. The data presented here suggest that S1 expression is limited to cells that are locked permanently in the nongrowing state, such as those of brain and muscle tissues, whereas EF-1α mRNA can be detected in all cells.

Materials and Methods

Materials. [^{32}P]UTP (650 Ci/mmol) was purchased from ICN Biochemicals Canada (Montréal, PQ); the pGEM plasmid and riboprobe synthesis system was from Promega (Madison, WI); digoxigenin-11-UTP and detection system was from Boehringer Mannheim Canada (Laval, PQ); all restriction enzymes were purchased from Pharmacia (Piscataway, NJ), except for BstNI from Stratagene (LaJolla, CA); rats were either Harlan Sprague Dawley (Indianapolis, IN) or Fischer 344 from the aging rat colony of the National Institute on Aging of the National Institutes of Health (Bethesda, MD).

S1 and rat EF-1α cRNA probe synthesis. The construction of the S1 (pS1-7) and EF-1α (pEF-1α-1) plasmid and synthesis of the 3' noncoding region cRNA was carried out as described (11). Minor modifications were added for the synthesis of digoxygenin-11-UTP labeled cRNA (19). Briefly, linearized plasmid (1 μg) was incubated for 1 hour at 37°C in a solution containing transcription buffer, 20 units of RNasin (RNase inhibitor), 10 μM dithiothreitol (DTT), GTP, ATP, CTP (500 μM each), unlabeled and digoxygenin-labeled UTP (250 μM each), and 40 units of T7 (antisense) or SP6 (sense) RNA polymerase. After the initial incubation, an additional 20 units of polymerase was added to the reaction mixture. The reaction was terminated by exhaustive DNase I digestion followed by ethanol precipitation. The reaction mixture was re-suspended in diethylpyrocarbonate (DEPC)-treated water. To verify that high-quality, digoxigenin-labeled full-length transcripts were synthesized using our method, trace amounts of [^{32}P]UTP (<1.0 μCi) was added to the reaction mixture. The transcription product was treated as described above and was analysed by 6% polyacrylamide/7 M urea gel electrophoresis. Exposure time of the gel to the autoradiogram was 2 hours.

Tissue preparation for *in situ* hybridization. The method used is essentially as described (19). Male Fischer 344 rats were decapitated, and their brains and livers quickly

removed and frozen on dry ice. Sections were cut at a thickness of 10-20 μm on a cryostat, thaw-mounted on poly-l-lysine-coated slides, and stored at -90°C for less than two days. On the day of the experiment, sections were fixed for 5 minutes in 3% paraformaldehyde made up fresh in 0.1 M PBS, and washed briefly in PBS.

Pre-hybridization. After fixation, sections were briefly washed in 2x SSC (saline sodium citrate), acetylated for 10 minutes in 0.25% acetic anhydride, 0.1 M triethanolamine (pH 8.0), rinsed in 2x SSC, rinsed in PBS, and incubated for 30 minutes in 0.1 M Tris/glycine buffer (pH 7.0). The sections were briefly washed in 2x SSC, dehydrated in ethanol, and allowed to air-dry.

Hybridization. Each section was covered with 100 μl of hybridization buffer containing 40% de-ionized formamide, 10% dextran sulfate, 1x Denhardt's solution (0.02% Ficoll, polyvinyl pyrolidone and bovine serum albumin each), 4x SSC, 10 mM DTT, 1mg/ml yeast total RNA, and digoxigenin-11-UTP labeled EF-1α or S1 cRNA. Hybridization was conducted at 45°C for 4 hours. After hybridization the slides were washed in 50% formamide containing 2x SSC at 50°C, treated with RNase A (20 μg/ml) in 2x SSC at 37°C for 30 minutes, rinsed in 2x SSC at room temperature, and incubated 15 minutes in 50% formamide, 2x SSC. The slides were left overnight in 2x SSC containing 0.05% Triton X-100 and 2% fetal calf serum at room temperature. For detection of signal, the sections were treated as described in the manufacturer's protocol (Boehringer Mannheim), but with a final dilution of the anti-digoxigenin-alkaline phosphatase-conjugated antibody at 1:2000.

Results

We have shown that S1 shares high amino acid sequence similarity with the known elongation factor EF-1α (1, 2). In our previous report, we demonstrated by RNase protection and Northern analysis the tissue-specific expression of S1 in brain, heart, and muscle (11). Using these two techniques we could not detect any signal in the other tissues that we have examined. To further verify S1 tissue specific expression, we performed in situ hybridization on brain and liver sections.

The probes that we used for in situ hybridization were the same as previously described (11). These probes are targeted to the 3' noncoding regions of S1 and EF-1α mRNA, which differ considerably in their sequence. The demonstration of probe specificity to their respective genes has been previously described in detail (11). In addition, because success of in situ hybridization is partially due to probe quality, we verified probe synthesis by adding trace amounts of [^{32}P]UTP to the reaction mixture, and performed polyacrylamide gel electrophoresis to analyze both newly synthesized cRNAs. Fig. 1 shows that digoxigenin-11-UTP can be incorporated into full-length transcripts with the described method; incorporation of digoxigenin-labeled UTP in the transcription product led to an increase in the size of the transcript, as observed in Fig. 1A. The presence of high amounts of digoxigenin-11-UTP and/or the absence of unlabeled UTP did not cause any major problem in transcript synthesis (i.e, polymerase pausing, wrong strand synthesis). Probe quality was also analysed by the direct detection method, as suggested by the manufacturer (Boehringer Mannheim; data not shown). The combination of both methods ensured that the cRNAs used in these experiments were full-length transcripts labeled to high specific activity.

Fig. 2 (and Fig.2; annex) shows representative results from in situ hybridization on liver sections. We did not detect any signal over background in any fields of liver

hybridized to digoxygenin-labeled S1 cRNA (Fig. 2A); in contrast, we detected very intense staining in the cytoplasm of all the cells when sections were hybridized with the EF-1α probe (Fig. 2B). The fact that the signal was mostly confined to the cytoplasm indicates specificity of hybridization. Sense control did give some nonspecific signal in certain cells, but the level of staining intensity was significantly lower than that observed for the EF-1α antisense probe (Fig. 2C). These results are in agreement with our previously published report (11), indicating the absence of signal for S1 and presence of abundant EF-1α transcript in liver.

In situ hybridization was also performed on different regions of rat brain. First, we detected a very strong signal in hippocampus and cortex, when sections were hybridized to either S1 (Fig. 3) or EF-1α (Fig. 4) cRNA, compared with sense control. Staining was observed in the hippocampus, specifically the Ammon's horn, the dentate gyrus, and in cortex, and was restricted to the cytoplasm of these cells (Fig. 4B). Areas of sections that are not stained correspond to the molecular layer, mainly long axonal extension and little content of cell bodies. The co-localization of both transcripts in this region of the brain may suggests that the expression of S1 and EF-1α is not mutually exclusive (i.e., EF-1α mRNA is present in S1-positive cells). Results obtained from the brain are also in agreement with our previous report, indicating the presence of high amounts of both transcripts in this tissue (11).

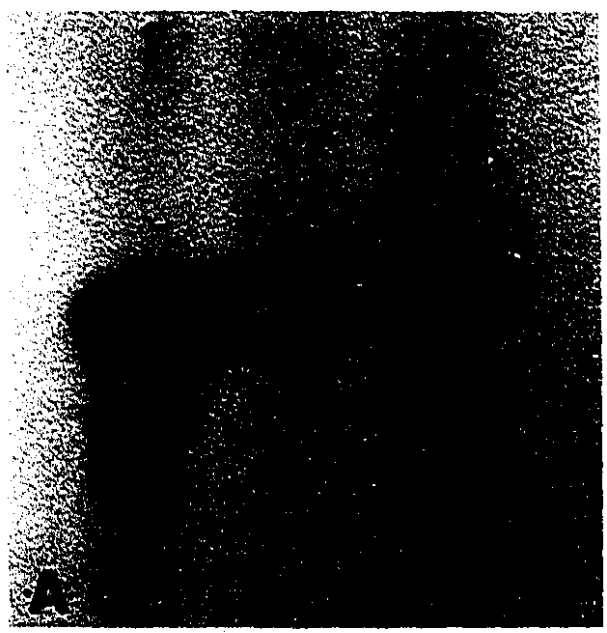
Finally, we pursue our analysis of S1 expression in two other regions of rat brain. In the cerebellum, the staining pattern suggests that S1 expression is limited to Purkinje cell bodies that border the granular and molecular layers; the signal observed was less intense than in the hippocampus region. We could also detect some strongly stained cells resembling motor neurons in the medulla, and anterior horn neurons in the spinal cord (data

not shown). These results suggest that S1 is express in different part of rat brain, and that certain neuron types are S1-positive.

Figure 1. Digoxigenin-UTP labeling of S1- and EF-1α-specific cRNAs.

(A) In vitro transcription of plasmid pS1-7 was performed as described in "Materials and Methods" in the presence of: (lane 1) no digoxygenin-11-UTP, 500 μM unlabeled UTP; (lane 2) 250 μM digoxygenin-11-UTP, 250 μM unlabeled UTP; and (lane 3) 500 μM digoxygenin-11-UTP, no unlabeled UTP. In each case, a small amount of [^{32}P]UTP (<1.0 μCi) was added to the reaction mixture.

(B) Synthesis of full-length S1 (lane 1) and EF-1α (lane 2) cRNAs. After incubation, an aliquot of each reaction mixture was run on a 6% urea/polyacrylamide gel. The gel was autoradiographed for 2 hours at -90°C.


A

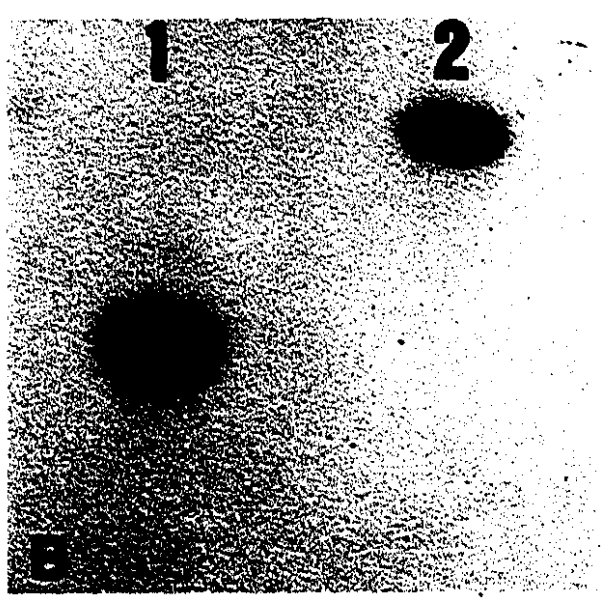

1
2
B

Figure 2. Cellular localization of S1 and EF-1α mRNA in rat liver.

Photomicrographs of liver sections hybridized with DIG-labeled (A) S1 antisense cRNA, (B) EF-1α antisense cRNA, and (C) EF-1α sense probe control. Arrows point at nuclei. Note that the dense signal for EF-1α antisense probe is confined to cytoplasmic regions of the cells. Bars = 25 μm.

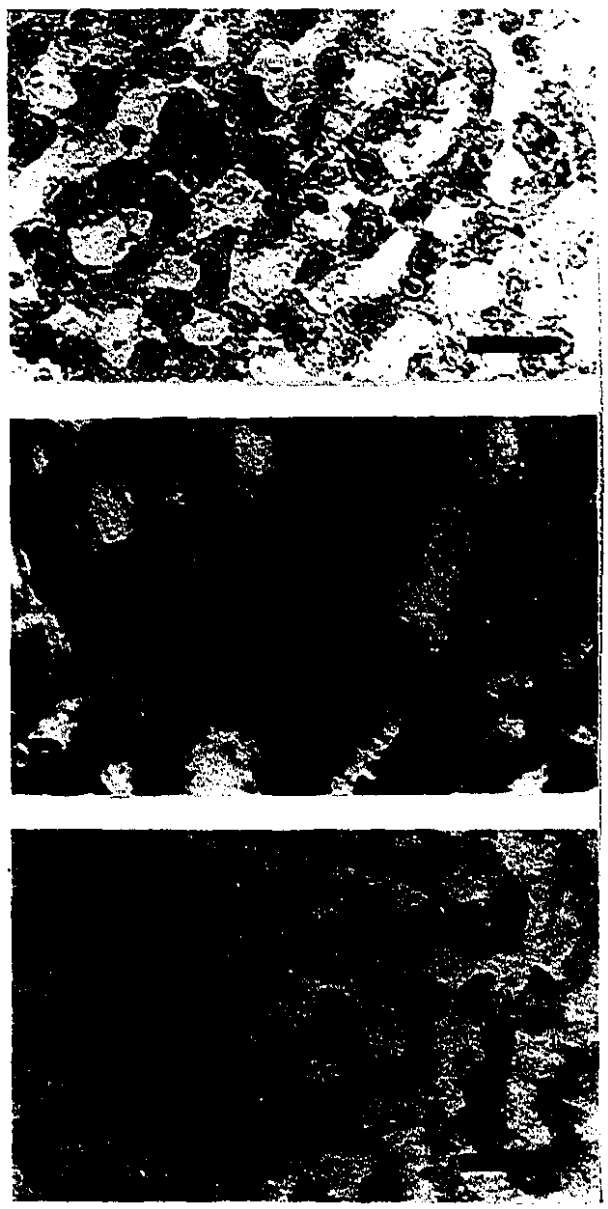


Figure 2. (Annex). Cellular localization of S1 and EF-1α mRNA in rat liver. Photomicrographs of liver sections hybridized with DIG-labeled (A) S1 antisense cRNA and (B) EF-1α antisense cRNA. Notice the total absence of S1-positive cells, whereas all cells are EF-1α positive.

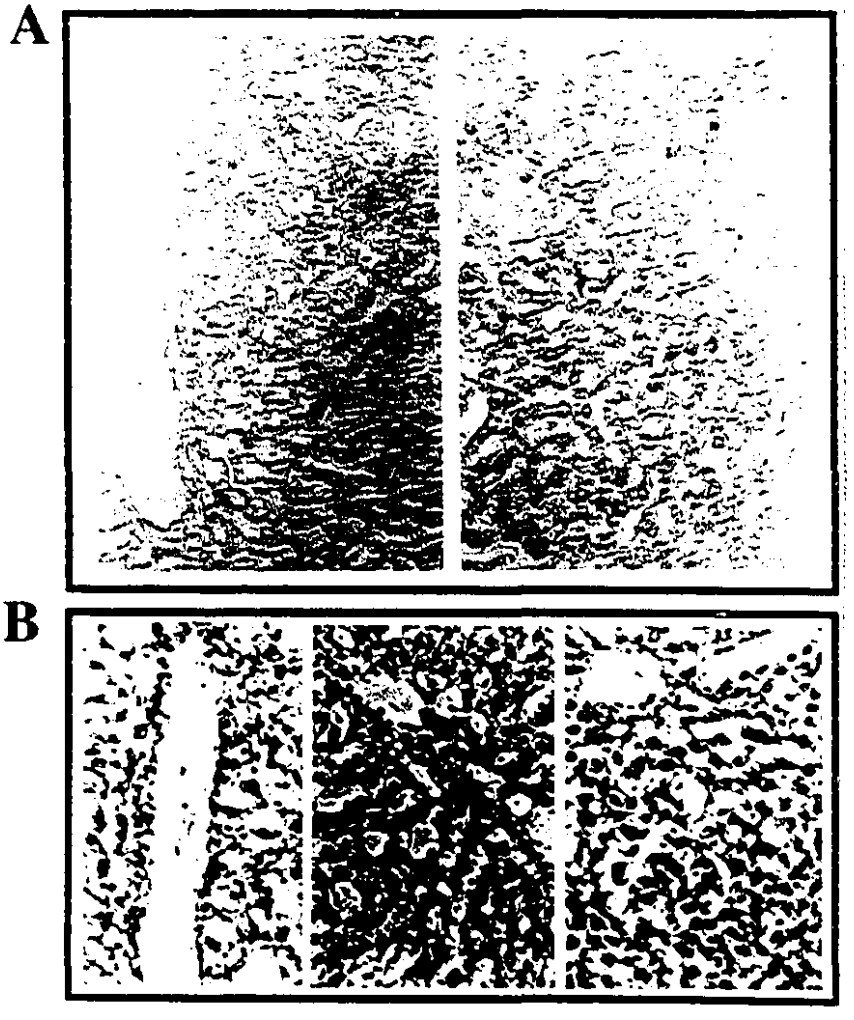

A
B

Figure 3. Cellular localization of S1 mRNA in the hippocampus region of rat brain. (A) Photomicrograph of a brain section showing areas of staining with DIG-labeled S1 antisense cRNA. Dark arrow indicates area of intense staining in the cortex, and open arrow points to staining in the hippocampus region (Ammon's horn). (B) Higher magnification of the same section as above. (C) Photomicrograph of a serial section hybridized to DIG-labeled control sense probe. Bar = 50 μm in each sections.


C

Figure 4. Cellular localization of EF-1α mRNA in rat brain.

(A) Photomicrograph of a section hybridized with DIG-labeled EF-1α antisense cRNA. Arrow points to the dentate gyrus, part of the hippocampus region. (B) Same section as above. Small arrows point to certain nuclei. (C) Another area showing the same degree of signal intensity with the EF-1α antisense probe. (D) Same type of section as C, reacted with control sense probe. Bars: (A) = 150 μm, (B) = 25 μm, (C) = 35 μm, (D) = 50 μm.

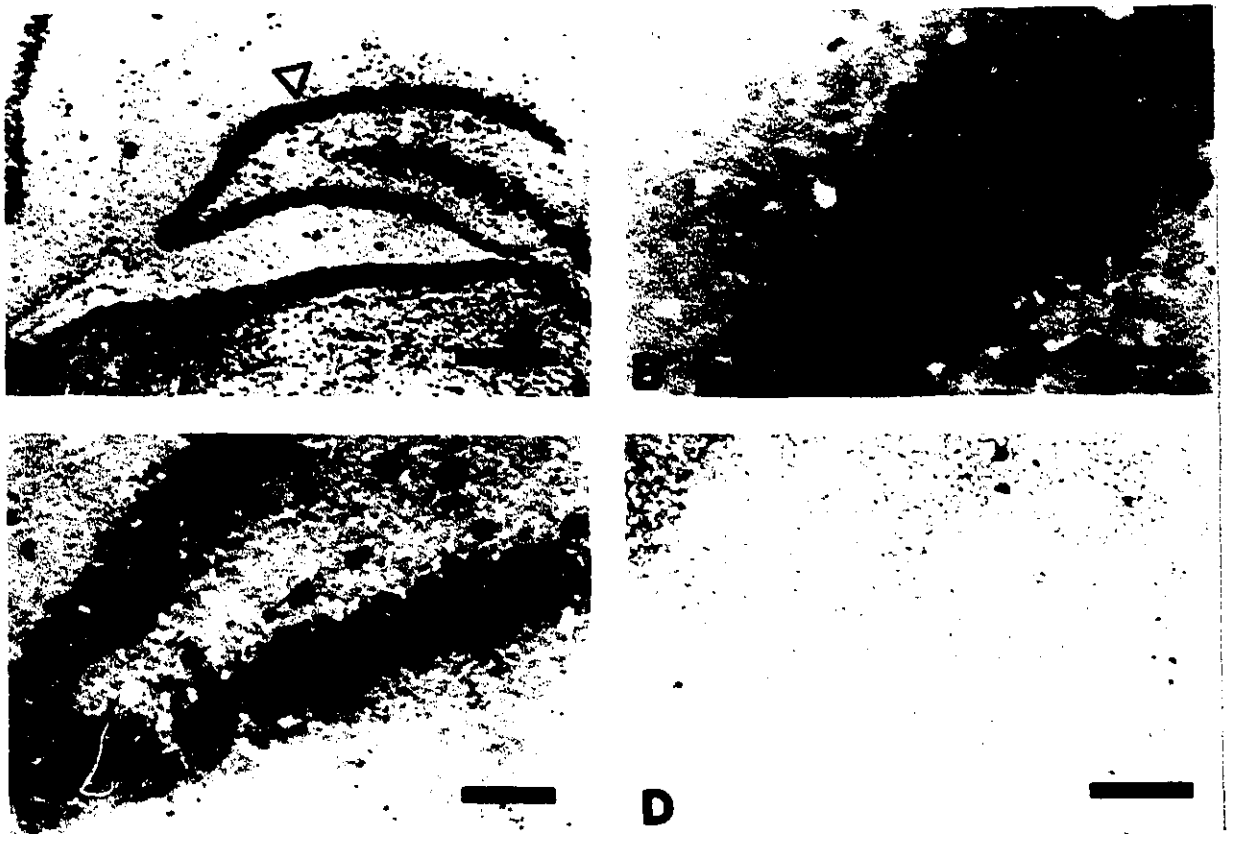
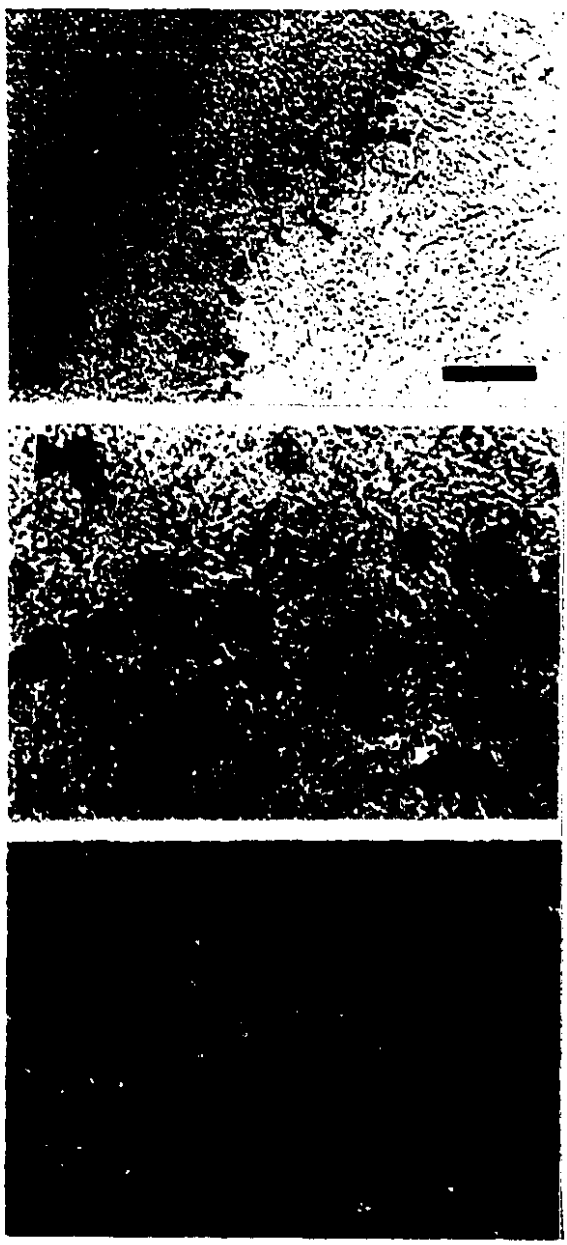

D

Figure 5. Cellular localization of S1 mRNA in the cerebellum and medulla regions of rat brain.

(A) Photomicrograph of the cerebellum hybridized to DIG-labeled S1 cRNA. Arrows indicate staining in the Purkinje cell layer between the molecular and granular layers of the cerebellum. (B) Photomicrograph showing staining in the medulla. (C) Higher magnification of B. Triangles in (B) and (C) depict strongly stained large cells resembling motor neurons. Bars: (A) = 60 μm, (B) = 25 μm, (C) = 15 μm.



Discussion

We report here the cellular distribution of S1 and EF-1α mRNA by in situ hybridization. We show that the non-radioactive digoxigenin system can be used to distinguish between two very similar mRNAs. In general, owing to the result of bad probe synthesis or very low incorporation of digoxigenin-11-UTP, the described DIG procedure does not always produce satisfactory results. Using this method with the addition of trace amounts of [^{32}P]UTP, we verify that the probes used are full-length and labeled to high specific activity. By verifying probe quality, we perform in situ hybridization with high reproducibility.

The results obtained by in situ hybridization are in agreement with our previous report describing S1 expression as limited to three tissues in mammals: brain, heart and muscle (11). We could not detect S1 mRNA in any liver cells, demonstrating that the absence of signal by Northern analysis and RNase protection is valid, and is not due to possible heterogeneous distribution of the S1 mRNA in that tissue. Taken together, these results confirm that the S1 gene is indeed not expressed at all in liver; in contrast, our results show that both tissues are positive for EF-1α mRNA. The presence of EF-1α in all cells that we have examined so far confirms our and other reports indicating the highly ubiquitous nature of the protein (4, 7, 9, 10, 11, 12, 13, 20, 22, 23).

In situ hybridization was also performed in different regions of the brain. Our results suggest that not all cells are S1-positive in the cerebellum and medulla regions of this tissue. However, it is difficult to precisely identify cell types by in situ hybridization. Thus, we feel that it is too early to confirm the exact nature of the cellular distribution of S1 in brain, but it seems that Purkinje cells, hippocampus neurons, and motor neurons of the brain stem are positive. We will be addressing this question by immunohistochemical studies using neuronal- and glial-specific antibodies and confocal microscopy technology.

The presence of S1 mRNA in brain, however, as detected by in situ hybridization, also confirms our previous finding that S1 mRNA is present in high abundance in this tissue.

The similar staining patterns that we observed for S1 and EF-1α mRNA in the hippocampus suggest that in certain cases EF-1α and S1 expression may overlap. This observation is in agreement with the muscle cell-culture system, where we detected EF-1α mRNA in myoblasts and myotubes, whereas S1 mRNA was only observed in the myotubes (data not shown). It is interesting that cells in the brain that already express high amounts of EF-1α transcript would also express S1 mRNA. The presence of S1 where there is abundant EF-1α suggests a possible novel way of regulating an aspect of the elongation stage of protein synthesis. Hence, an increase in the cellular pool of active EF-1α could result in enhanced translation fidelity, as shown in yeast (18), and also in decreased translation efficiency and growth rate, as reported experimentally (14, 17, 21) and as predicted by computer simulation (16) of protein translation mechanisms. Further studies will deem necessary to demonstrate the co-localization of both transcripts and proteins in certain cells. Nonetheless, the results obtained by in situ hybridization are consistant with our hypothesis that mammalian species contain a tissue-specific EF-1α isoform. Yet, there are several instances in which EF-1α and EF-1α-like proteins have been shown to be associated with actin (25), the mitotic spindle (15), and RNP (6). Because of these observations, we remain open to the possibility that S1 possesses some function other than the traditional role of EF-1α in protein synthesis; rather, it may prove to be associated with some aspects of control mechanisms that determine the program of terminal differentiation in muscle and brain.

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CHAPTER 4

The results presented in chapter 2 and 3 strongly suggest that S1 mRNA is present in only brain, heart, and muscle. These tissues are composed of cells that permanently withdraw from the cell cycle early during development (i.e., neurons, cardiomyocytes, and myocytes). This led us to hypothesize that S1 gene expression was terminal differentiation-dependent and specific for these cell types. The data presented in the previous chapter agree with our hypothesis; they suggest that certain types of neurons are S1 positive, whereas all liver cells are S1-negative. Still, to demonstrate the validity of our hypothesis, the expression of S1 mRNA must correlate with terminal differentiation in the latter three tissues during their in vivo development.

The following study was designed to confirm our hypothesis that S1 is a terminal differentiation-dependent EF-1α-like gene. Several questions were asked: (1) What are the structural similarities between the putative rat pS1 and EF-1α? (2) Are the S1 and EF-1α mRNAs transcribed by one or several genes in the rat genome? (3) Does S1 mRNA accumulate to levels similar to those of EF-1α in S1-positive tissues? (4) What is the pattern of expression of S1 and EF-1α mRNA during rat in vivo development and in culture myogenesis? The publication presented in chapter 4 is as it appears in the literature (Lee et al., **1993** J. Biol. Chem., 268, 24453-24459). Table 1 and an extra panel were joined to Fig. 5, as annexes to lend support to the conclusion that S1 expression is dependent upon myotube formation during myogenesis in culture.

DIFFERENTIAL EXPRESSION OF S1 AND ELONGATION FACTOR-1 ALPHA DURING RAT DEVELOPMENT.

Abstract

Elongation factor-1 alpha (EF-1α) is a highly conserved protein functioning in peptide elongation during mRNA translation. A cDNA, S1, was isolated; its deduced amino acid sequence shares high similarity with mammalian EF-1αs (92%). While EF-1α mRNA is present in all tissues, S1 mRNA can only be detected in brain, heart, and muscle. We report here that the retropseudogene phenomenon is attributable to EF-1α and not S1, the latter being represented by a single copy in the rat genome. The S1 steady-state mRNA level is consistently higher than EF-1α in S1-positive tissues. S1 mRNA can only be detected late during brain, heart, and muscle development in vivo, and increases to a plateau in early post-natal life. In a cultured muscle system, S1 expression is dependent upon the formation of myotubes, although the accumulation of S1 mRNA is significantly lower than that observed in adult skeletal muscle. EF-1α mRNA levels are downregulated during brain, heart, and muscle development, but stay relatively steady in liver. We show here that EF-1α and S1 are differentially expressed during rat development, and that the activation of S1 gene expression is subsequent to the terminal differentiation process in brain, heart, and muscle.

Introduction

It is becoming increasingly evident that translational factors are directly implicated in the regulation of gene expression (reviewed in Ref. 1). While there is ample evidence that translation initiation factors are important in regulating peptide synthesis, recent reports have pointed to a direct contribution by elongation factors (reviewed in Refs. 2-6). One of the most studied proteins implicated in peptide elongation is the elongation factor-1 alpha (EF-1α). EF-1α is part of the EF-1 complex, which includes EF-1β, γ, and δ, and promotes GTP-dependent binding of aminoacyl-tRNA to the A site of ribosomes, in a codon-dependent manner, during peptide synthesis. It is a highly conserved, abundantly expressed protein that has been identified in all procaryotic and eucaryotic species studied so far.

In an effort to better understand the cellular role of EF-1α, investigators have uncovered a wide variety of structurally related genes (7-17). These different EF-1α genes undergo strict cell type-specific or stage-specific regulation of expression, as reported in Saccharomyces cerevisiae (12), Mucor racemosus (13), Artemia salina (14), Drosophila melanogaster (15) and Xenopus laevis (16-18). It is in the latter two species that most of the developmental work has been produced. Drosophila has two copies of the EF-1α gene (F1 and F2). Both genes are differentially expressed during early development, but only F1 mRNA can be detected in adult tissues (15). The situation is similar in Xenopus laevis where three active EF-1α genes have been characterized so far (42Sp50, EF-1αO, and EF-1αS); all of the genes are active in early development, while only EF-1αS is expressed after neurulation and in the adult (16, 17). No studies on the developmental regulation of EF-1α gene expression have been reported in mammalian models so far.

We recently reported the identification of a second EF-1α-like gene, S1, in rat and mouse (19, 21). S1 shares high (92%) amino acid sequence similarity with mammalian

EF-1α, but differs in its 3'-UTR and in its expression pattern; EF-1α mRNA can be found in all tissues, while S1 mRNA can only be detected in brain, heart, and muscle (20-22). The fact that S1 gene expression is limited to these three tissues has led us to hypothesize that S1 is a terminal differentiation-specific EF-1α-like protein, whose expression is limited to irreversibly growth-arrested cells such as neurons, myocytes and cardiomyocytes.

As a first step to test this hypothesis, we investigated the developmental regulation of S1 and EF-1α mRNA in rat. We report here that S1 and EF-1α are independent genes under different developmental control. S1 mRNA can only be detected late during neurogenesis and myogenesis in vivo, and accumulates to attain a plateau early after birth, at the time when terminal differentiation is completed. Furthermore, S1 expression is dependent upon the formation of myotubes during myogenesis in culture. The EF-1α gene is regulated in a way that corresponds more to the need for protein synthesis in developing and adult tissues. These results are consistent with our hypothesis, and suggest that mammalian species contain an EF-1α-like gene, S1, the expression of which is regulated in a terminal differentiation-dependent manner during development.

Experimental Procedures

Materials. [^{32}P]UTP (650 and 3000 Ci/mmol) and [^{32}P]dCTP was purchased from ICN Biochemicals Canada (Montréal, Québec); the pGEM plasmid and riboprobe synthesis was obtained from Promega (Madison, WI). All restriction enzymes were purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ), except for BstNI, which was purchased from Stratagene (LaJolla, CA). Hybond nylon filters were purchased from Amersham Corp (Arlington Heights, IL). Rats were obtained from Harlan Sprague-Dawley (Montréal, Québec).

Construction and purification of S1 and EF-1α gene-specific probes. The construction of the EF-1α and S1 probes, and the demonstration of their specificity to their respective genes, has been reported elsewhere (21). pEF-1α-1 was digested with SspI restriction enzyme in order to produce a 235-nucleotide cRNA containing 175 nucleotides from the 3'-UTR of rat EF-1α mRNA. pS1-7 was digested with BstNI restriction enzyme to produce a 107-nucleotide cRNA containing 99 nucleotides from the S1 mRNA 3'-UTR. cRNA synthesis was performed using the manufacturer's protocol (Promega, WI) in the presence of T7 polymerase and [^{32}P]UTP. For RNase protection, S1 cRNA was subsequently purified by acrylamide gel electrophoresis, as described elsewhere (21). LK295 is a 136bp cDNA, containing the 3'-UTR of human α-actin mRNA, that was kindly provided by Dr. L. Kedes from the University of Southern California (Los Angeles, CA).

Tissue preparation and total RNA isolation. Pregnant Harlan Sprague-Dawley female rats were obtained at gestation day 11, and this was used as our reference for developmental staging. Tissues were isolated from only three embryos/female at different time points during gestation, to limit RNA degradation. Total RNA was isolated from tissues and cells using the acidic phenol/guanidium thiocyanate procedure as described (23), followed by a phenol/chloroform extraction and ethanol precipitation. Quality and

quantity of the RNA was evaluated by UV spectrophotometry, and by denaturating agarose gel analysis in the presence of ethidium bromide.

Cell cultures. The rat L8 myoblast cell line, originally isolated by David Yaffe (24) from the skeletal muscle of early postnatal Wistar rats, was obtained from the American Type Culture Collection (ATCC; #CRL1769). L8 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (proliferating medium; PM). Cells were kept at low cell density by frequent passage. To promote fusion, cells were grown to confluence and transferred to DMEM supplemented with 2% horse serum (differentiation medium; DM). Multinucleated myotubes were detected within 12 hours after the cells were transferred to DM, and the fusion process was completed within 48 hours. Other cell lines, including normal rat kidney cells (NRK) and rat smooth muscle cells (A7), were cultured in DMEM, 10% fetal calf serum unless indicated otherwise.

Southern analysis. Chromosomal DNA was extracted from Sprague-Dawley rat liver using standard protocols (25). Ten µg of DNA was digested overnight with various restriction enzymes, resolved by electrophoresis through a 1.0% agarose gel, and transferred to a nylon membrane. Prehybridization of the filters was performed for 4 hours at 58°C in a solution containing 6x SSC (1x SSC= 0.15 M NaCl and 0.015 M sodium citrate), 5x Denhart's solution (1x Denhart= 0.02% (w/v) each polyvinylpyrrolidone, bovine serum albumin, and Ficoll), 100 µg/ml yeast total RNA, and 10 µg/ml denatured salmon sperm DNA. The [^{32}P]UTP-labeled cRNAs were added to a final concentration of 2-3 x 10^5 cpm/ml, and hybridization was carried out overnight. After the removal of the probe, the final wash was performed in a solution containing 1.0x SSC, 0.1% SDS at 60°C. Membranes were processed for autoradiography at -90°C.

Northern analysis. Total RNA was run on a 1.2% agarose gel containing 7% formaldehyde, transferred to a nylon membrane, and processed for prehybridization and

hybridization as described above. Membranes were processed for autoradiography at -90°C.

RNase protection assay. Protection experiments were performed essentially as described, with certain modifications (21, 25). Total RNA was hybridized in the presence of 2-3 x 10^5 cpm of pEF-1α1-1 and/or pS1-7 cRNA in a solution that contained 80% formamide, 1mM EDTA, 0.4 M NaCl, and 40 mM 1,4-piperazinediethanesulfonic acid (PIPES) for 16 hours at 50°C. The hybridization mixture was digested with 40 µg/ml RNAse A when the pS1-7 probe was used alone. The pEF-1α-1 cRNA contains several stretches of A and U nucleotides that form duplexes that can "breathe", causing internal cleavage by RNase A and degradation of the protected fragment (26). In order to avoid this problem, RNase A was omitted when the pEF-1α-1 probe was present in the hybridization mixture. RNase T1 was used alone at a concentration of 2 µg/ml. The digestion reaction was carried out for up to two hours, especially when the EF-1α signal was strong, to reduce background. High incubation time with RNase T1 did cause partial degradation of the protected S1 fragment, as seen in the experiments with hindlimb skeletal muscle where we detected two protected fragments (Fig. 4B) that migrated closely together. After proteinase K digestion, samples were processed for ethanol precipitation and run on a 6-8% polyacrylamide/7 M urea gel. Autoradiograms of the gels were performed at -90°C.

Densitometry. Autoradiographs were scanned with an LKB densitometer and analyzed with the Gel Scan XL computer program software (Pharmacia LKB Biotechnology Inc.). Scanning was performed on autoradiographs exposed for different times, to assure that the reading was in the linear range of the different signals on the films. Signals were also quantified by excising and counting radioactivity in the appropriate gel slice by liquid scintillation counting. Results obtained were standardized to the number of UTP nucleotides in each probe (23 UTPs for pS1-7, and 56 UTPs for pEF-1α-1). Scanning

was not necessarily performed on the autoradiographs that are presented in the different figures.

Results

Comparisons of the amino acid sequence between rat S1 and EF-1α of different species. We have reported that S1 shares high sequence similarity to mammalian EF-1αs (19-21). Fig. 1 shows the amino acid sequence alignment between S1 and EF-1αs of different species. In all cases, the well characterized functional domains are either highly or totally conserved between S1 and the EF-1αs. Most substitutions are of a conservative nature, and are found in the less conserved regions. While we do not know the exact function of S1, the sequence similarity in the highly conserved domains suggests that it may share common characteristics with EF-1α, such as the capacity to bind GTP and aminoacyl-tRNA.

Southern hybridization analysis of rat chromosomal DNA. Mammalian genomes are known to contain several copies of the EF-1α gene; however, a majority of these are retropseudogenes (27). In the present study, we asked whether: (1) S1 was a unique member of the EF-1α gene family, and (2) the retropseudogenes originated from S1 or EF-1α mRNA. Southern analysis was performed on rat total genomic DNA. Blots were hybridized with S1 (pS1-7) or EF-1α (pEF-1α-1) specific cRNA targeted to the 3'-UTR of both mRNAs. The demonstration of the specificity of each probe has been previously reported (21). Several bands were detected using the EF-1α probe, while only one band was observed with the S1 probe (Fig. 2). The size of the detected bands for S1 correspond to the molecular weight predicted by the restriction map of the S1 gene, S10 (19). These results suggest that the retropseudogenes have originated from EF-1α mRNA. The S1 gene is represented by only one copy in the rat genome, and does not have any retropseudogenes.

Figure 1. Comparison of rat S1 deduced amino acid sequence with that of EF-1αs from different species.

The amino acid sequences are predicted from the cloned cDNAs. Rat S1 (19); rat EF-1α (11); EF-1αS, Xenopus laevis adult somatic form (17); EF-1αO, Xenopus laevis germ cell-specific form (17); F1, Drosophila melanogaster adult form (15); F2, Drosophila melanogaster pupal stage-specific form (15); TEF-1 ; first EF-1α gene to be isolated from Saccharomyces cerevisiae (51). Dashed lines indicate identity; dots are introduced to maximize the alignments. Characterized GTP-binding domains are denoted as GTP-1 to 4. Highly conserved domains between the different eucaryotic EF-1αs are denoted as conserved regions 1-6. Notice that the majority of the amino acid substitutions between the EF-1αs of the different species are localized outside of these two latter domains. This observation also holds true for rat S1 and rat EF-1α.

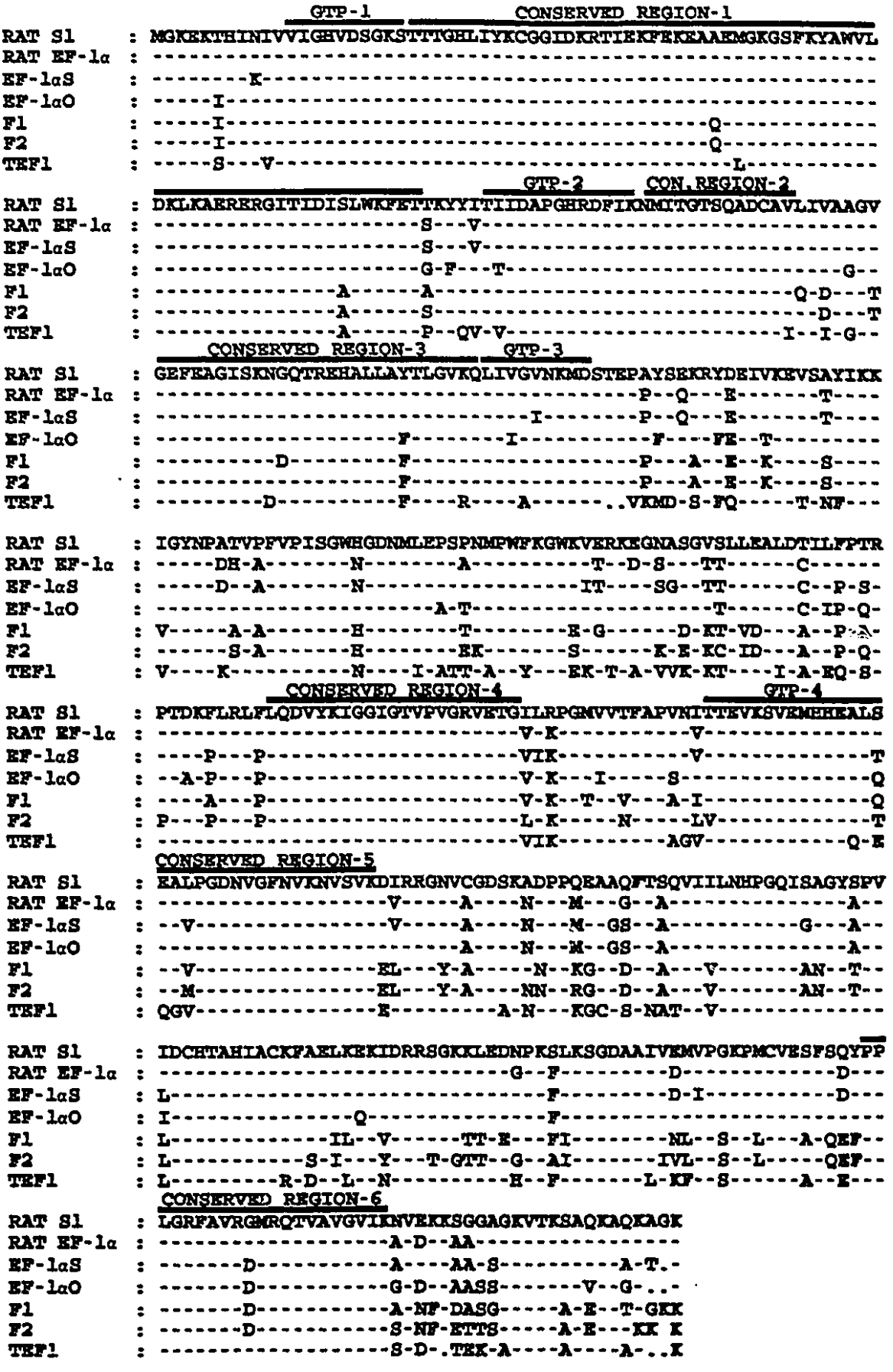
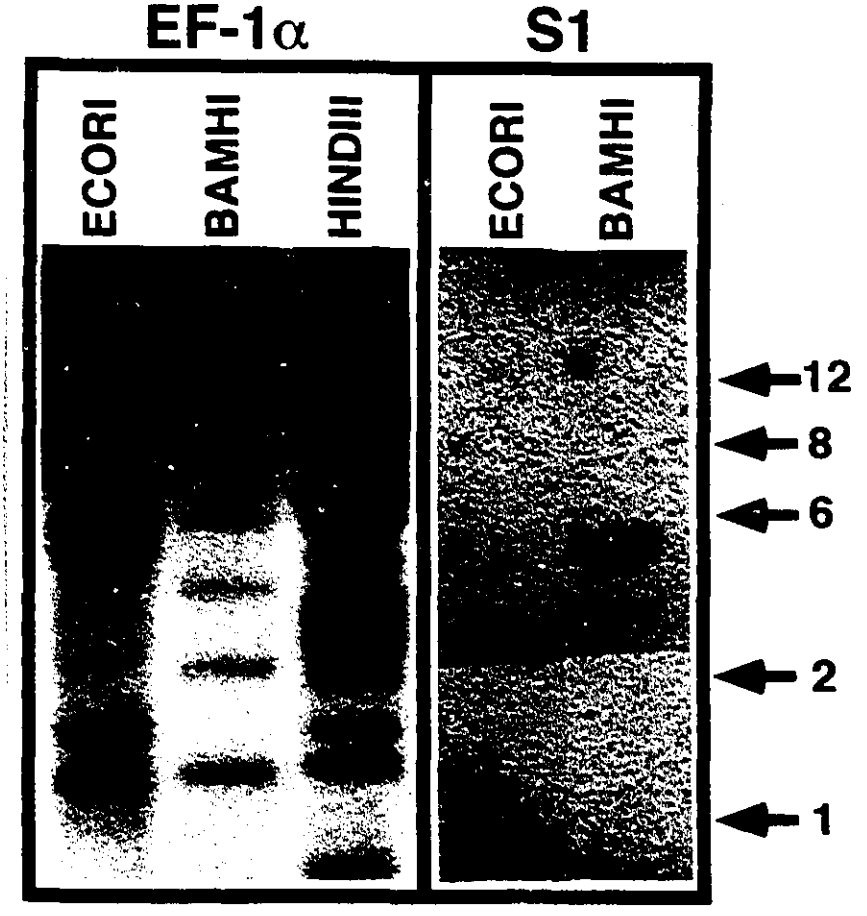

GTP-1
CONSERVED REGION-1
GTP-2
CON.REGION-2
CONSERVED REGION-3
GTP-3
CONSERVED REGION-4
GTP-4
CONSERVED REGION-5
CONSERVED REGION-6
RAT S1
RAT EF-1α
EF-1αS
EF-1αO
F1
F2
TEF1

Figure 2. Southern hybridization analysis of rat EF-1α and S1 genes.

Rat liver genomic DNA was digested with different restriction enzymes (EcoRI, BamHI, HinDIII, subjected to electrophoresis on a 1.0% agarose gel, transferred to a nylon membrane, and hybridized as described under "Experimental Procedures" with either EF-1α (pEF-1α-1) or S1 (pS1-7) specific 3'-UTR [^{32}P]UTP-labeled cRNA probes. The size markers are given in kilobases. The contrast in the granularity of the bands observed is due to the difference in time of exposure: 16h for EF-1α and 5 days for S1. Note the multitude of EF-1α bands, compared to the single S1 bands with either enzyme.


EF-1α
S1
ECORI
BAMHI
HINDIII
ECORI
BAMHI
12
8
6
2
1

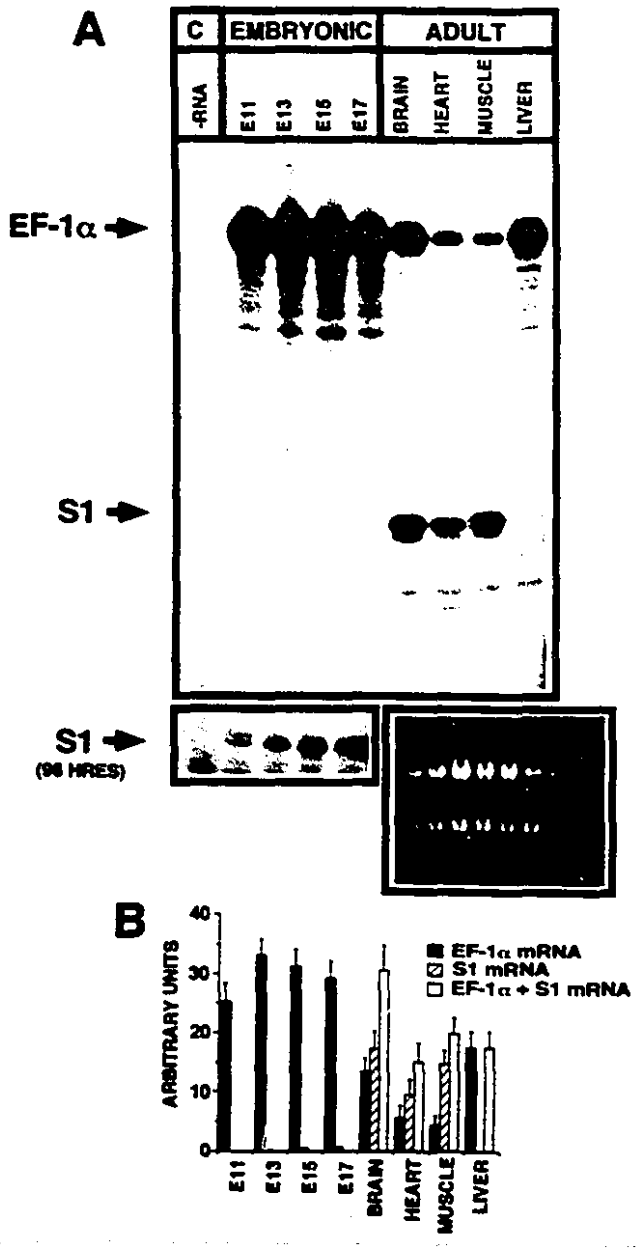
S1 and EF-1α mRNA levels in rat embryo and adult tissues. EF-1α mRNA can be found in very high abundance in cells of every organism studied thus far. We wanted to compare the S1 mRNA levels to those of EF-1α in S1-positive tissues. Steady-state levels of both S1 and EF-1α transcript were measured in different 6-month-old rat tissues and compared to their respective levels in embryos, either by RNase protection (Fig. 3A) or by Northern analysis (data not shown). Densitometry was performed on the autoradiographs; and the results obtained are represented as a graph in Fig. 3B. The amount of EF-1α transcripts is significantly higher in developing rat embryos compared to those observed in adult tissues, and stays relatively constant throughout embryonic life. EF-1α message levels are lower in S1-positive tissues compared to those observed in liver or other S1-negative tissues such as spleen and kidney (data not shown). Brain and muscle have about the same amount of S1 mRNA, and approximately twice the amount detected in heart. In all cases, S1 mRNA is more abundant than EF-1α mRNA in S1-positive tissues. Very low but gradually increasing levels of S1 transcript could be detected in developing total embryo RNA. The low level of signal is due to the fact that S1 mRNA distribution is limited to only certain tissues in developing embryos. These results indicate that S1 mRNA accumulates to very high levels, comparable to those of EF-1α, in S1-positive tissues.

S1 and EF-1α expression during rat development. The S1-positive tissues share at least one common trait: a certain populations of cells are permanently locked in a state of nonproliferation, i.e., neurons, myocytes, and cardiomyocytes. If indeed S1 mRNA is present only in terminally differentiated cells, its mRNA should be first detected only at the point when cells permanently leave the cell cycle, upon terminal differentiation, during brain, heart, and muscle development. To test this hypothesis, we analyzed EF-1α and S1 mRNA level in brain, heart, muscle, and liver in developing and

Figure 3. Comparison between EF-1α and S1 mRNA levels in embryonic and adult tissues.

A) RNase protection analysis. Total RNA (10 μg) from developing rat total embryos or 6-month-old adult tissues was hybridized with either pEF-1α-1 or pS1-7 [^{32}P]UTP-labeled cRNAs, and RNase protection was carried out as described under "Experimental Procedures." The ethidium bromide staining of the sample RNA is presented in the lower right part of panel A to illustarte RNA quantity and quality. Autoradiography was performed for 8 hours. Also shown is a 96 hour exposure time for the first five lanes of S1 in developing embryos (S1 (96hres)).

B) Band intensity evaluation of autoradiograph A along with other similar data not presented. Standard deviation from the mean of three experiments is shown. Values are in arbitrary units. E, embryonic day.


A
C
EMBRYONIC
ADULT
-RNA
E11
E13
E15
E17
BRAIN
HEART
MUSCLE
LIVER
EF-1α
S1
S1
(96 HRES)
B
ARBITRARY UNITS
40
30
20
10
0
EF-1α mRNA
S1 mRNA
EF-1α + S1 mRNA
E11
E13
E15
E17
BRAIN
HEART
MUSCLE
LIVER

postnatal rats (Fig. 4). First, Northern analysis was performed with the EF-1α probe as shown in Fig. 4A. Surprisingly, we noticed that EF-1α expression was regulated in a different manner in the four tissues. No significant changes were observed during liver development. In S1-positive tissues, EF-1α expression was down-regulated, but at different times during development. EF-1α mRNA levels are higher in embryonic brain (day 20) compared to their newborn and adult counterparts. The most dramatic decrease is in muscle, where levels of EF-1α are diminished by 95% within the first month of postnatal life. This down-regulation of EF-1α expression is not a rapid phenomenon, taking more than two weeks to attain the final levels seen in the adult. This observation is not specific to muscle, as we observed the same general pattern in heart. In both muscle and heart, EF-1α down-regulation coincides with the termination of the myogenic process.

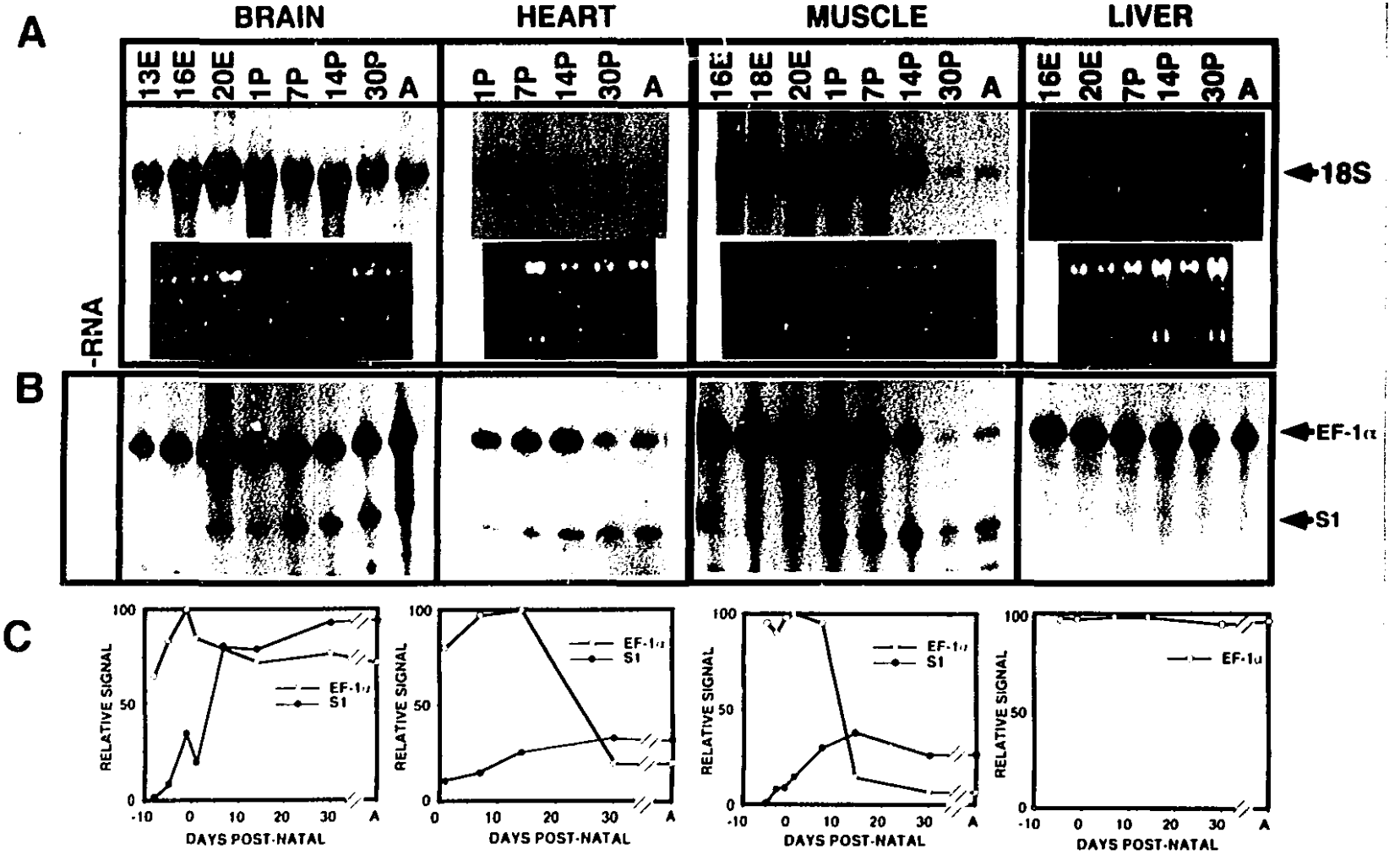
To study S1 expression, we preferred an RNase protection assay in order to detect low amounts of transcripts, particularly because our previous Northern analysis showed barely detectable signal in rat embryonic brain (data not shown) . Both S1 and EF-1α probes were used for this assay, the latter serving as an adequate internal control whose signal we compared to that obtained with Northern analysis (Fig. 4A). Results presented in Fig. 4B were obtained from a totally different set of rats, and are reported as graphs in Fig. 4C. For EF-1α, similar pattern of expression were obtained by RNase protection assay (Fig. 4B) compared to the Northern analysis (Fig. 4A). S1 mRNA was detected late during brain and muscle (embryonic day 16-20) development, and steadily increased to attain a plateau early during postnatal life. In muscle, the S1 signal decrease observed between postnatal days 14 and 30 is due to a loading artifact (EF-1α signal is significantly lower in figure 4B lanes 30 days postnatal and adult compared to the same lanes in Fig. 4A, but the S1/EF-1α ratio is similar). We initiated our study in heart late during development,

Figure 4. S1 and EF-1α expression in developing rat tissues.

A) Northern blot analysis of EF-1α expression. Total RNA (5 μg) isolated from brain, heart, muscle, and liver was subjected to electrophoresis through a formaldehyde denaturating 1.2% agarose gel, and after Northern blotting, hybridized with pEF-1α-1 specific probe. Ethidium bromide staining of the RNA is shown. Exposure time of the autoradiographs is approximately 16 hours.

B) RNase protection analysis of S1 expression. Total RNA (5 μg) from a totally different set of embryos was hybridized with both pEF-1α-1 and pS1-7 cRNA, and RNase protection assay was carried out as described under "Experimental Procedures." We preferred this method in order to increase our detection capacity of the S1 transcript. EF-1α signal was compared to Fig. 4A and used as a control; similar signals were detected in both Northern and protection analyses, indicating that the increase in S1 signal was not due to loading artifacts, except for muscle, lane 30P (see "Results"). Autoradiography was performed for 8 hours, except for muscle (6 hours).

C) Band intensity evaluation of the autoradiograms. The signals on the autoradiograms were quantitated by densitometry, and the results are presented as graphs. In the abscissa, -10 denotes days before birth. Because of gel migration and loading artifacts, Brain A has been estimated from data previously published (21). Signal for Muscle 30P has been estimated from the signal obtained with the EF-1α Northern blot (panel A). E, embryonic day; P, postnatal day; A, 6-9-month-old adults.


A
BRAIN
HEART
MUSCLE
LIVER
13E 16E 20E 1P 7P 14P 30P A
1P 7P 14P 30P A
16E 18E 20E 1P 7P 14P 30P A
16E 20E 7P 14P 30P A
18S
-RNA
B
EF-1α
S1
C
RELATIVE SIGNAL
DAYS POST-NATAL
EF-1α
S1

because of the difficulty in isolating this organ without contamination with other tissues in early embryos. We observed a small but significant increase in S1 mRNA levels in heart between postnatal days 1 and 30. As with our previous aging study, we did notice a certain variability in our data that is probably due to variations expected when dealing with individual animals. Nevertheless, the increase in S1 mRNA levels does seem to correlate in time with the terminal differentiation process which occurs in brain, heart, and hindlimb muscle cells. No signal was detected at any time in liver, suggesting that S1 expression is limited to the above mentioned three tissues even during development. In all cases, S1 and EF-1α mRNA levels remained steady after 1 month. These results are in agreement with our previous report, demonstrating that S1 and EF-1α gene expression are independent of the aging process in these four tissues (21).

S1 and EF-1α expression during myogenesis in culture. To define precisely the kinetics of S1 expression during terminal differentiation, we set out to determine if we could reproduce S1 and EF-1α in vivo expression in a myogenic culture system. To do so, we used the rat L8 cell line originally described by Yaffe and co-workers (24). L8 are mononucleated myoblasts which, upon reaching confluence, can readily fuse to form multinucleated myotubes in the presence of the appropriate concentration of serum. This process is thought to recapitulate in vivo myotube formation at the cellular and molecular level (reviewed in Ref. 28 and 29). We noticed the formation of multinucleated myotubes as early as 12 hours after cells were transferred to differentiation medium (DM). In our hands, approximately 70% of the nuclei were incorporated within myotubes in the first 48 hours after the transfer to DM (Fig. 5A). RNase protection assays, using the S1 and EF-1α probes, were performed on RNA extracted from L8 cells at different time points during the myogenic process (Fig. 5B and 5C). We detected S1 transcripts only after the fusion process was completed (48-96 hours after the transfer to DM). S1 mRNA levels

Figure 5. S1 and EF-1α expression during L8 myogenesis in culture.

A) Microphotographs of L8 cells incubated in different serum conditions. The illustrations also serve as an appropriate morphological control for the presence or absence of myotubes. See "Experimental P ocedures" for cell culture details. Arrowheads points to myotubes. No increase in fusion was detected after 48 hours in differentiation medium (DM). HS: horse serum; FCS: fetal calf serum; PM: proliferation medium. B) RNase protection assay of S1 expression during L8 myogenesis. Total RNA (50 µg each) isolated from L8 or NRK cells cultivated in either PM or DM for different times, and adult skeletal muscle (Adult; 1 µg), was hybridized to pS1-7 [^{32}P]UTP-labeled cRNA and subjected to RNase protection assay. The arrow labeled PROBE points to the unprotected probe that can be detected after a longer exposure time of the autoradiograph, and can also be detected in the two negative controls (-RNA, or yeast RNA; 50 µg). Exposure time of the autoradiograph was 96 hours. C) RNase protection assay of EF-1α expression during L8 myogenesis. Total RNA (5 µg for each lane) was hybridized to pEF-1α-1 [^{32}P]UTP-labeled cRNA and subjected to RNAse protection. Autoradiography was performed for 12 hours. D) Northern blot analysis of α-actin expression. Five µg of total RNA was subjected to electrophoresis on a denaturating 1.2% agarose gel, and processed for Northern blot analysis with 3'-UTR α-actin specific [^{32}P]dCTP-labeled cDNA (LK295). Gel staining of the analyzed RNA is also presented in the bottom panel. -RNA, hybridization in the absence of RNA; YEAST, hybridization in presence of yeast total RNA; PM SPARSE, proliferating L8 myoblasts; PM CONFLU, confluent L8 myoblasts; ADULT, 6-month old rat skeletal muscle tissue; NRK DM, NRK cells incubated for 96 hours in DM; PM 96 HRS, L8 cells incubated in proliferation medium for 96 hours.

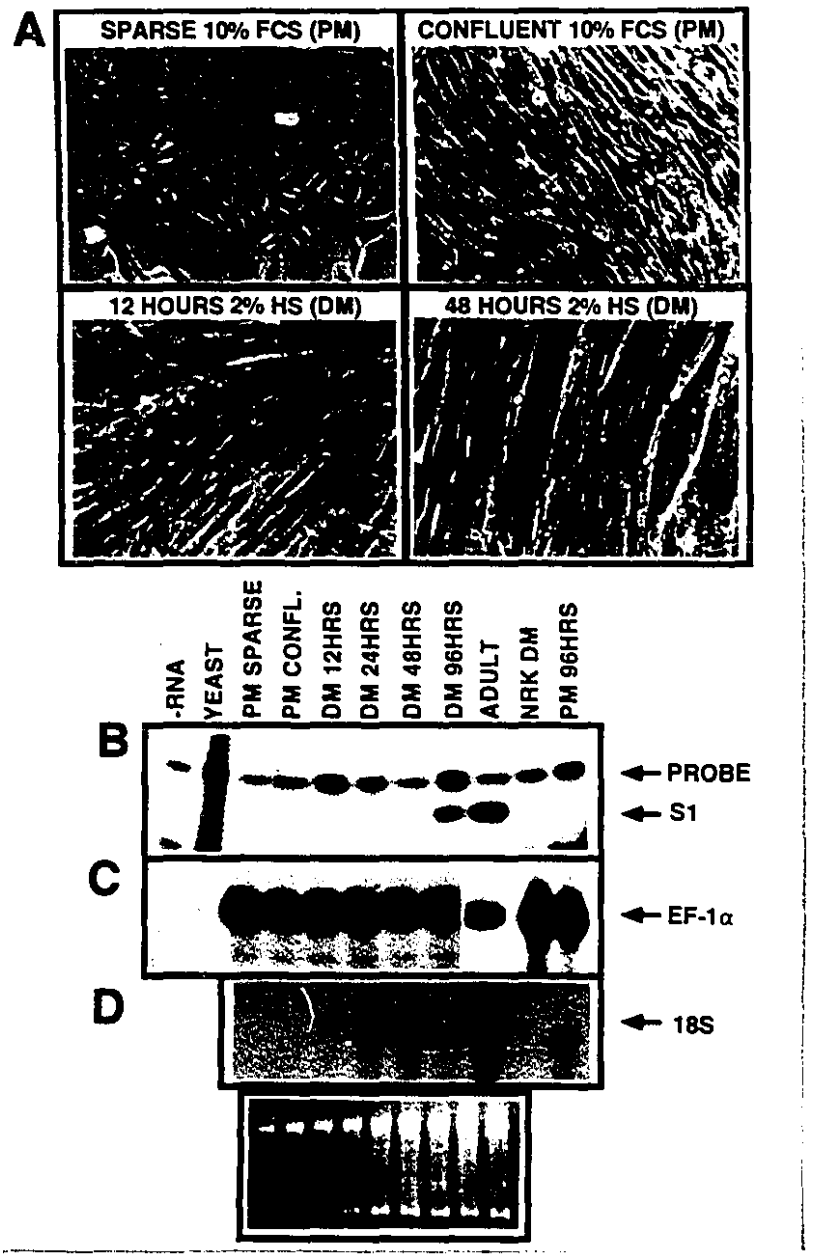
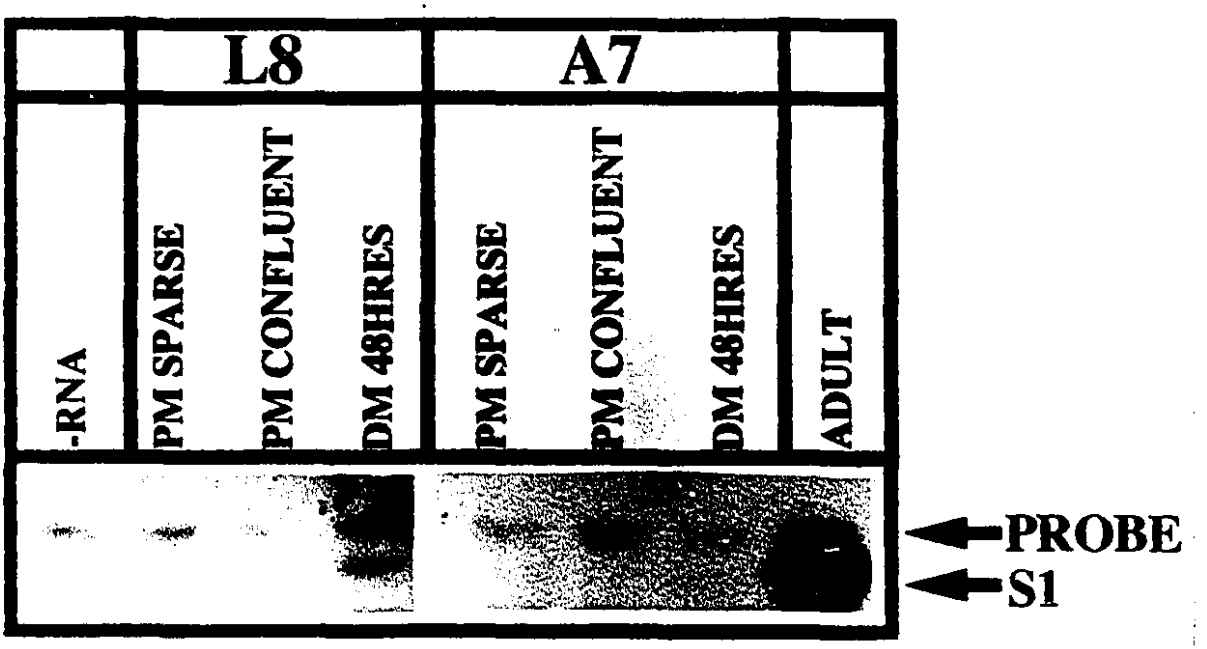

A
SPARSE 10% FCS (PM)
CONFLUENT 10% FCS (PM)
12 HOURS 2% HS (DM)
48 HOURS 2% HS (DM)
-RNA
YEAST
PM SPARSE
PM CONFL.
DM 12HRS
DM 24HRS
DM 48HRS
DM 96HRS
ADULT
NRK DM
PM 96HRS
B
PROBE
S1
C
EF-1α
D
18S

Figure 5. (Annex). S1 expression during myogenesis in culture.

RNase protection assay of S1 expression in L8 skeletal muscle and A7 smooth muscle cells in different culture conditions (20 µg each except adult, 5 µg). Autoradiography was performed for 48 hours. -RNA: hybridization in the absence of RNA; PM SPARSE: proliferating L8 or A7 myoblasts; PM CONFLUENT: confluent L8 or A7 myoblasts; DM 48 HRES: L8 or A7 cells incubated for 48h in differentiation medium; ADULT: 6-month old rat skeletal muscle tissue. Notice the absence of signal in the smooth muscle cells in DM.


L8
A7
-RNA
PM SPARSE
PM CONFLUENT
DM 48HRES
PM SPARSE
PM CONFLUENT
DM 48HRES
ADULT
PROBE
S1

detected in the L8-cultured myotubes were significantly lower compared to those observed in adult skeletal muscle (Fig. 5B). However, discrepancies in the levels of expression of different genes between muscle cell culture systems versus muscle tissues in vivo have been reported (30, 31). Transfection experiments with the S1 gene (S10) promoter have indicated that the low level of S1 mRNA is probably due to a certain transcriptional repression in these cells (data not shown). Detectable levels of S1 mRNA were also obtained with fused mouse C2C12 cells (data not shown). Howerver, A5r7 rat smooth muscle cells were S1-negative, as were several other nonmyogenic cell lines, when treated in the same culture conditions as L8 cells (see Fig 5 and Table 1; annexes). In contrast to the situation observed in vivo, EF-1α mRNA levels stayed unchanged during the myogenic process. RNA extracted from the same time points was analyzed with a specific cDNA to the 3'-UTR of the α-actin isotype mRNA. Signal corresponding to the α-actin transcript was detected in confluent cultures within 12 hours after the addition of DM. These results are in agreement with several published reports indicating that the expression of α-actin is an early event during myogenesis in culture (31-33). To verify whether the S1 signal detected in the fused myotubes was not due to culture conditions such as cell confluence or serum deprivation, RNase protection assay were performed on total RNA isolated from L8 cells that were kept in proliferation medium (PM) for 96 hours (very low amount of myotubes were detected) or in a series of cultures including rat NRK cells, and smooth muscle cells (Fig. 5; annex) that were incubated in DM for 96 hours (figure 5B, C, D, Table 1; annex, and data not shown). Absence of detectable signal indicated that myotubes must be formed in order to detect S1 mRNA. Although levels are relatively low, S1 expression in culture does correlate with the in vivo work, inasmuch as in both cases, S1 mRNA can only be detected late during myogenesis.

Table 1

List of all cell lines analysed with the S1- and EF-1α-specific probes

Cells	Description	Probe	RNA[1]	Northern analysis[2]			RNase protection assay		
			(μg)	Sparse	Confl.	DM	Sparse	Confl.	DM
L8	Rat skeletal	pS1-7	T-50	ND[3]	ND	ND	-	-	+
	muscle	pEF1α	T-50	ND	ND	ND	+++	+++	+++
L6	Rat skeletal	pS1-7	T-50	ND	ND	ND	-	-	+/-
	muscle	pEF1α	T-50	ND	ND	ND	+++	+++	+++
C2	Mouse skeletal	pS1-7	T-50	ND	ND	ND	-	-	+
	muscle	pEF1α	T-50	ND	ND	ND	+++	+++	+++
NRK	Normal rat	pS1-7	T-50	ND	ND	ND	-	-	-
	kidney	pEF1α	T-50	ND	ND	ND	+++	+++	+++
	fibroblasts	PS1-7	A-20	-	-	-	-	-	-
		pEF1α	A-20	+++	+++	+++	+++	+++	+++
3T3	Mouse swiss	pS1-7	T-50	ND	ND	ND	-	-	-
	3T3 fibroblasts	pEF1α	T-50	ND	ND	ND	+++	+++	+++
		pS1-7	A-20	-	-	-	-	-	-
		pEF1α	A-20	+++	+++	+++	+++	+++	+++
A7	Rat smooth	pS1-7	T-50	ND	ND	ND	-	-	-
	muscle	pEF1α	T-50	ND	ND	ND	ND	ND	+++
1213	Rat skin	pS1-7	A-10	ND	ND	-	ND	ND	-
MG63	Human	pS1-7	T-20	ND	ND	ND	-	-	ND
	transformed	pEF1α	T-20	ND	ND	ND	++	++	ND
	fibroblasts	S1olig	T-10	-	-	ND	ND	ND	ND
		HT7-3	T-10	++	++	ND	ND	ND	ND

[1] T, total RNA; A, poly(A)+ RNA.

[2] See Material and Methods for procedures.

[3] N D, not determined; -, no signal; +/-, baerly detectable signal; +, low signal; ++, strong signal; +++, very strong signal

Discussion

S1 is structurally related to the different members of the EF-1α gene family. We report here the characterization of the in vivo expression of S1 and EF-1α in rat. While we do not know the exact function of S1, the high sequence similarity that it shares with EF-1αs of different species suggests that S1 may bind GTP and aminoacyl-tRNAs. S1 and EF-1α differ in their amino acid sequences, but the substitutions are localized primarily in the non-conserved regions. Such differences between the different members of the EF-1α gene family have also been observed in other species (i.e., Xenopus EF-1αS and EF-1αO, Drosophila F1 and F2). Work in lower species has shown that the amino acid differences have not led to an important modification in function. For example, in Xenopus, the three characterized EF-1αs are known either to participate directly in protein synthesis, or at least to possess the capacity to bind GTP and tRNAs (18, 34). However, one cannot simply infer that S1 participates directly in protein synthesis, especially in light of reports relating EF-1α and similar proteins to functions other than peptide elongation (35-40).

The rat EF-1α retropseudogenes originate from the EF-1α mRNA and not S1 mRNA. As with other housekeeping genes, the mammalian EF-1α gene family is mostly composed of inactive retropseudogenes. Retropseudogenes are mainly thought to arise from a reverse copy of an original mRNA, which is then shuttled to the germ cell genome (41). Our results strongly suggest that in rat, the retropseudogene phenomenon arises from the EF-1α mRNA and not the S1 mRNA. These results are in agreement with a recently published paper showing that in Xenopus, the retropseudogenes also originate from the somatic, adult form of EF-1α, and not the tissue-specific or germ line-specific forms (42). We have shown that S1 is an unique gene in the rat genome. Hence, the rat genome contains at least two active members of the EF-1α gene family. This conclusion is

probably true for mammalian species in general, considering that we have isolated mouse[1] and human[2] homologues of rat S1 cDNA.

EF-1α gene expression is tightly regulated during development. In other species, the developmental expression of EF-1α genes is an early embryonic event. The developmentally-regulated EF-1α genes are thought to play a role in stage-specific regulation of elongation, probably by binding specific tRNAs or other components of the translational apparatus, or by increasing the pool of EF-1α necessary to maintain a high rate of peptide synthesis in metabolically active, developing tissues. In rat, this function seems to be accomplished by the EF-1α gene itself (not S1), the expression of which is up-regulated in certain developing tissues compared to their adult counterparts. The adult tissues also contain significantly different amounts of EF-1α mRNA. The regulation of the EF-1α gene probably reflects the different needs for protein synthesis in the developing and adult tissues. It is not surprising that the EF-1α gene is so highly regulated, considering recent reports demonstrating that the overexpression of EF-1α renders mammalian cells more susceptible to oncogenic transformation (43), and that EF-1α levels are upregulated in cancer cells in vivo (44-45).

S1 gene expression is a late event during myogenesis and neurogenesis. S1 gene expression is up-regulated at a point in time when cells (neurons, cardiomyocytes, and myocytes) are permanently leaving the cell cycle during neuro- and myogenesis. S1 mRNA levels, as well as the EF-1α/S1 mRNA ratio, stay relatively constant from early postnatal life and onward. In muscle and heart, the EF-1α/S1 ratio is mainly accomplished by a general down-regulation of EF-1α transcripts. In brain, EF-1α mRNA levels stay relatively the same, and the EF-1α/S1 ratio is mostly the result of an increase in S1 mRNA levels. The differential expression of S1 and EF-1α probably reflects the cellular and biochemical heterogeneity of the mentioned tissues. In hindlimb muscle development, as

well as in the L8 culture system, S1 expression is significantly preceded by the expression of known myogenic markers including, MyoD1, myogenin, and α-actin (46-48). These markers can be detected in high abundance as early as embryonic day 11, or early after the transfer of myoblasts to differentiation medium (DM) (28, 29, 31-33, 46-48). The fact that S1 expression is a late event during the myogenic process, as well as during brain and heart development, suggest that S1 functions as part of a postdifferentiation program. This notion is consistent with the hypothesis that any cellular differentiation state is an active phenomenon requiring continuous regulation (review in Ref. 49 and 50). We suggest that S1 plays a role, as part of a general mechanism, in maintaining neurons, cardiomyocytes, and myocytes in a state of permanent growth arrest.

Possible role of S1 and its relationship to terminal differentiation. S1 mRNA accumulates to very high levels, comparable to those of EF-1α, in S1-positive tissues. This is a significant phenomenon, considering that the EF-1α promoter can stimulate in vitro transcription to higher levels than can the adenovirus major late promoter (9). However, this raises the question: why do these cell types need S1 if they are already abundantly expressing EF-1α (3-5% of the total protein content in brain)? One hypothesis is that S1 may compete with EF-1α for GTP and aminoacyl-tRNA, thus reducing the elongation rate, and protein synthesis, which may be necessary for keeping cells in a nongrowing state. An alternative explanation is that terminally differentiated cells contain different components of the translation machinery, such as specific tRNAs and translational factors. S1 may function in a fashion similar to that of EF-1α, specific for these cell types. Nevertheless, we do not rule out the possibility that the putative S1 function bears no relationship with the involvement of EF-1α in protein synthesis. This last consideration is based on several reports that EF-1α and EF-1α-like proteins are part of the mammalian valyl-tRNA synthetase (35), and also complex with messenger ribonucleoproteins (36), the mitotic apparatus (37), and actin (38). We are presently preparing monoclonal antibodies

specific to both S1 and EF-1α, to start investigating the cellular role of S1. In all, the fact that S1 expression is a late event during embryogenesis is consistent with our hypothesis that S1 is a terminal differentiation-specific EF-1α-like gene, whose expression is subsequent to the completion of brain, heart, and muscle development.

1- Lee, S., and Wang, E. Unpublished results

2- Lee,S., Ann, D., and Wang, E. Unpublished results

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CHAPTER 5

The EF-1α protein has been totally conserved in mammals. This characteristic of EF-1α prompted us to hypothesize that S1 is also present in mouse and man. We also hypothesized that the residue differences between rat S1 and EF-1α have led to functional distinctiveness for S1. If so, then these changes would have been conserved in mammals. To demonstrate those hypotheses, we have cloned the mouse and human homologues of rat S1.

The following study was designed to confirm our hypotheses We asked two questions: Is there an S1 gene in mouse and man? Is the putative pS1 primary amino acid sequence totally conserved between rat, mouse, and human? To answer these questions, we have cloned the mouse and human homologues of rat S1. The results presented in chapter 5 are part of a manuscript submitted for publication (Lee, S. et al., Gene). An extra panel was added to figure 1 as an annex to support our conclusions.

CLONING AND CHARACTERIZATION OF cDNAs CODING FOR THE MOUSE AND HUMAN HOMOLOGUES OF RAT S1.

Abstract

We recently reported the cloning of a cDNA (S1) in rat whose deduced amino acid sequence shares high similarity with that of mammalian elongation factor-1 alpha (EF-1α), a protein involved in the binding of aminoacyl-tRNA to the ribosome during peptide synthesis. S1 differs from EF-1α in its 3' UTR and expression pattern; S1 mRNA can only be detected in brain, heart, and muscle, whereas EF-1α mRNA can be found in all tissues. We report here the isolation of a full-length cDNA from a mouse brain library and a partial cDNA from a human hippocampus library who share extensive sequence homology to that of rat S1. We show that, as with mammalian EF-1αs, the predicted primary amino acid sequences of rat, mouse, and man S1 are totally similar, except for one conservative substitution. The 5' and 3' untranslated regions of the mammalian S1 mRNA are also conserved. These results indicate that mouse and human contain a second member of the EF-1α gene family, the S1 gene. They also suggest that our result obtained in rat may be extrapolated to mouse and man.

Introduction

Elongation factor-1 alpha (EF-1α) is a ubiquitous protein that is involved in the elongation step of protein synthesis (reviewd in Ref. 1). EF-1α cDNAs have been isolated from several species such as Escherichia coli (2), Saccharomyces cerevisiae (3), Drosophila melanogaster (4), and Xenopus laevis (5). In mammals, EF-1α cDNAs have been cloned in mouse (6), rat (7, 8), rabbit (9), and man (10, 11). The predicted amino acid sequences from mammalian EF-1α cDNAs are either highly or totally similar. For example, rabbit EF-1α is 100% similar to human EF-1α, whereas mouse and rat EF-1α are about 98% similar. The EF-1α mRNA 3' UTR is also highly conserved in mammals.

We recently reported that rat contains a second EF-1α-like gene, S1 (12). The deduced amino acid sequence of the cloned S1 cDNA shares high similarity (92%) to that of rat EF-1α, confirming its identity as a member of the rat EF-1α gene family (7). However, S1 differs from EF-1α in its 3' UTR and expression pattern; EF-1α mRNA can be found in all cells and tissues that we have studied so far, whereas S1 mRNA can only be detected in certain neurons in brain, in heart, in skeletal muscle, and in culture myotubes (13-15).

The cloning of the S1 cDNA in rat and the identification of its mRNA in mouse has led us to hypothesize that the S1 gene is present in all mammalian species. We also hypothesized that the residue differences between rat S1 and EF-1α have resulted in functional distinctiveness for S1, and that these changes have been conserved in mammals. In this report, we describe the isolation and characterization of cDNAs for the mouse and human homologues of rat S1. We show that, as with EF-1α, the S1 putative protein has been totally conserved, except for one conservative amino acid substitutions, between rat, and man. The 5' and 3' UTRs of mammalian S1 mRNA are also highly similar. The

results presented in this report are consistent with our hypothesis, and suggest that our observation in rat may be extrapolated to mouse and man.

Materials and Methods

Materials. [^{32}P]-UTP (650 Ci/mmol) was purchased from ICN Biochemicals Canada (Montréal, PQ, Canada); the pGEM plasmid and Riboprobe synthesis system were obtained from Promega (Madison, WI); all restriction enzymes were purchased from Pharmacia (Piscataway, NJ), except BstNI from Stratagene (LaJolla, CA). The human hippocampus cDNA library and the mouse brain cDNA library were also purchased from Stratagene. All DNA sequencing was performed using [^{35}S]dATP and Sequenase.

Construction and synthesis of rat S1-specific probe. Rat S1 cDNA was subcloned into pGEM-3z EcoRI site and designated pS1-7 (13). pS1-7 was digested with the BstNI restriction enzyme to produce a cRNA of 107 nucleotides, containing 99 nucleotides of the S1 3' UTR. cRNA synthesis was carried out using the protocol of the manufacturer in the presence of [^{32}P]UTP and T7 RNA polymerase, followed by exhaustive DNase I digestion and ethanol precipitation.

Total RNA isolation. Because of the difficulty of obtaining fresh human tissues, we opted to verify probe specificity to human S1 using total RNA isolated from skeletal muscle obtained from patients undergoing surgery. Human skeletal muscle, as well as human MG63 cell, total RNA was isolated using the acidic phenol/guanidium thiocyanate procedure, followed by a phenol/chloroform extraction and ethanol precipitation. Quantity and quality of the isolated RNA were evaluated by UV spectrophotometry and by denaturing agarose gel electrophoresis.

RNase protection and Northern analysis. Both procedures were performed as described elsewhere (13)

Cloning and sequencing of mouse and human S1 cDNA. The human hippocampus cDNA library and the mouse brain cDNA library were obtained from Stratagene (LaJolla, CA). The library was screened with the pS1-7 [^{32}P]UTP-labeled cRNA by plaque hybridization, carried out at 60°C. Positive clones were analyzed by restriction mapping and Southern analysis with the pS1-7 probe. The clones that contained the largest insert, referred to as pMB-S1 and pHH-S1-1, were further characterized. A nest of unidirectional deletion inserts, from either end separately, was generated by exonuclease III and S1 nuclease digestion, as described previously (Ann et al., 1991). DNA sequences were determined by the dideoxy chain termination method using [α-^{35}S]dATP and Sequenase with universal primers, reverse primer, or synthetic oligonucleotide primers predicted from the sequence of the opposite strand. All DNA sequence were confirmed by sequencing both strands, and nucleotide sequences were analysed using MBIR software provided by the University of Minnesota Microbiology Computer Group.

Results

The first step consisted of the screening of appropriate mouse and human cDNA libraries, with the pS1-7 probe. This probe is targeted to the 3' UTR of rat S1 and can distinguish between S1 and EF-1α mRNA in rat, mouse (13), and human (Fig.1). We choose the human hippocampus cDNA library and the mouse brain library based on our in situ hybridization and RNase protection results that indicated a strong signal in rat hippocampus and mouse brain (13-14). No signal was obtained when we screened other cDNA libraries (Fig.1; annex). Several positive clones were isolated from both library. We choose one clone from the mouse library, termed pMB-S1, that contained an insert of about 1.8 kb. All the clones isolated from the human library had inserts of small size (<800 bp) except one, termed pHH-S1, that contained an insert of about 1.6 kb. Those two clones were selected for subsequent analysis.

The pMB-S1 cDNA is composed of 1808 bp, and contains only one long open reading frame of 1389 nucleotides coding for 463 amino acids, with the start translation codon at position 136 followed by a stop translation signal at position 1525 (Fig. 2). The stop signal is followed by 117 bp of high GC-rich sequence, a poly(A) signal (AATAAA) at position 1783 and a small poly(A) track at the 3' end of the clone. Nucleotide alignment of pMB-S1 has shown that the putative 5' and 3' UTRs are 90% homologous to those of rat S1, but are totally different from those of mammalian EF-1α (Table 1).

The pHH-S1-1 cDNA is composed of 1585 bp, with only one long open reading frame of 1305 nucleotides coding for 435 amino acids, followed by a stop translation signal at position 1304 (Fig. 3). The stop signal is followed by 117 bp of high GC-rich sequence, a poly(A) signal at position 1565 and a small poly(A) track at the 3' end of the clone.

Nucleotide alignment of pHH-S1-1 putative 3' UTR indicates that it is over 90% homologous to that of rat S1, but is totally different from that of human EF-1α (Table 1).

The pMB-S1 and pHH-S1 cDNAs contain one large open reading frame, and the amino acid sequences derived therefrom are aligned to the amino acid sequence of rat S1 and human EF-1α (Fig. 4). The alignment indicates that pMB-S1 and pHH-S1 are totally similar to rat S1 except for one conservative amino acid difference (A->S) at position 331 between rat and human S1. As with rat S1, most of the amino acid substitutions between mouse S1, human S1, and EF-1α are located in clusters, whereas the other parts of the molecules are totally similar. Important amino acids, implicated in GTP-binding and association with aminoacyl-tRNA, are also conserved between mouse and human S1, and EF-1α (Table-1). pHH-S1 cDNA does not contain the first 28 amino acids in the N-terminal region of the protein, indicating that it is a partial cDNA. These 28 amino acids are part of the first region implicated in the binding of GTP, and are the most conserved sequences between bacterial EF-Tu/eucaryotic EF-1α, and rat S1 (15). It is thus very likely that the first 28 amino acids of human S1 are the same as those of rat S1 and eucaryotic EF-1αs.

Figure 1. Detection of S1 mRNA in human skeletal muscle.

A) RNase protection analysis. Total RNA (10 μg) from rat and human skeletal muscle, as well as human MG63 fibroblasts, was hybridized to pS1-7 [^{32}P]UTP-labeled cRNA (PROBE), and RNase protection was carried out as described in "Materials and Methods." Autoradiography was performed for 8 hours. The two arrows point to the protected probe. -RNA: probe incubated in the absence of RNA. Note that the protected probe in the human sample (2) is smaller than that of rat (1). This is probably due to the nucleotide differences in the 3' UTR of rat and human S1 mRNAs.

B) Northern blot analysis. Total RNA (10 μg) isolated from human skeletal muscle was subjected to electrophoresis through a formaldehyde denaturing 1.2% agarose gel, transfered to a nylon filter, and hybridized with the pS1-7 [^{32}P]UTP-labeled cRNA. Exposure time of the autoradiograph was 16 hours. Note the single band with molecular weight to that of rat and mouse S1.

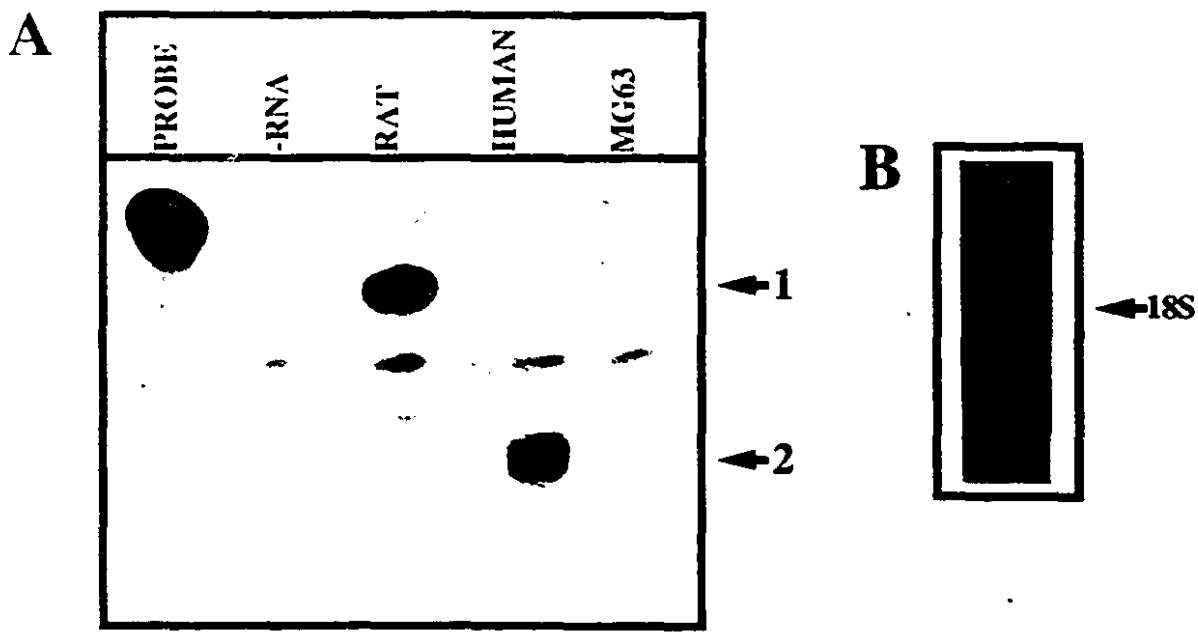

A
PROBE
-RNA
RAT
HUMAN
MG63
1
2
B
18S

Figure 1 (Annex). Screening of different human cDNA libraries with the rat pS1-7 cRNA.

A) Hippocampus library. B) MG63 transformed fibroblast library. C) WI-38 normal fibroblast library. D) Aorta library. See "Materials and Methods" for procedures. Notice that specific signal is only detected in the hippocampus library.

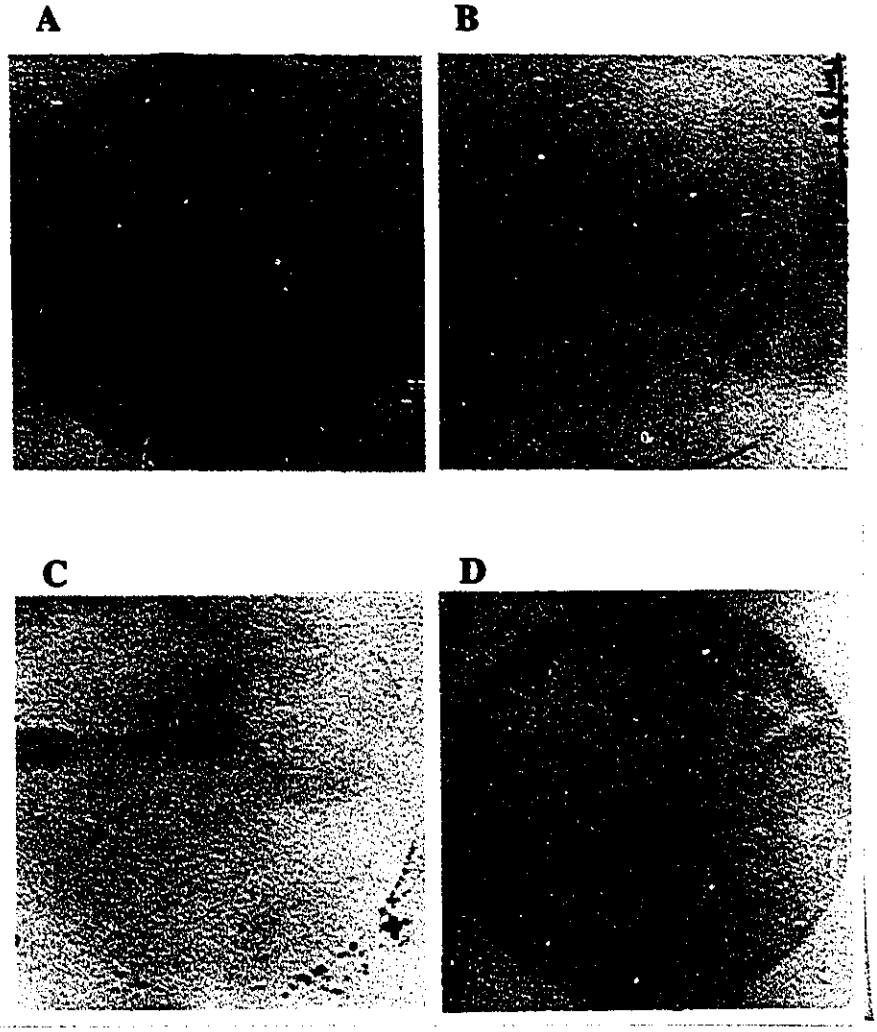

A
B
C
D

Figure 2. Nucleotide sequence of the pMB-S1 insert. The putative start and stop codon, and the poly(A) addition signal, are underlined. Notice the GC-rich sequence after the stop codon that is characteristic of the S1 mRNA.

```
1     TGGAGAGATAAAACCGCCGGCGCCGGCCACCAGTCCTTCTGACTGAGTCCTCGGCGCTGG  60
61    AGTTCCTGACCAGCAATATACCTCAACCCCAAACCAGAGCCCCCACAGTGCCAGCCCCTC  120
121   CCTCACCCAGGCAGAATGGGCAAGGAGAAGACACACATCAACATTGTGGTCATTGGCCAC  180
181   GTGGACTCAGGCAAGTCCACCACGACAGGCCACCTCATCTACAAGTGTGGTGGCATCGAC  240
241  .AAGCGGACCATCGAGAAGTTTGAGAAGGAGGCAGCAGAGATGGGGAAGGGCTCTTTTAAA  300
301   TATGCCTGGGTGCTGGACAAGCTGAAGGCCGAGCGGGAACGGGGCATCACCATCGACATC  360
361   TCCCTCTGGAAGTTTGAGACCACCAAGTACTACATCACCATCATCGATGCTCCCGGCCAC  420
421   CGAGACTTCATCAAGAATATGATTACAGGCACATCCCAGGCCGACTGTGCAGTACTGATC  480
481   GTCGCGGCGGGCGTGGGAGAGTTTGAGGCGGGCATCTCCAAGAAGGGGCAAACCCGGGAA  540
541   CAGGCACTCCTGGCCTACACTCTGGGTGTGAAGCAGCTCATTGTGGGTGTCAACAAGATG  600
601   GACTCCACGGAACCAGCCTACAGCGAGAAGCGCTATGATGAGATTGTTAAGGAGGTCAGC  660
661   GCCTACATCAAGAAGATCGGCTACAACCCAGCCACGGTGCCCTTCGTGCCCATCTCGGGC  720
721   TGGCATGGTGACAACATGCTGGAACCTTCACCTAATATGCCATGGTTCAAGGGCTGGAAA  780
781   GTAGAGCGTAAGGAAGGAAATGCAAGCGGCGTGTCCCTGCTGGAAGCCCTGGACACAATC  840
841   CTGCCCCCCACCCGCCCCACTGAAAAGCCCCTTCGTCTGCCTCTGCAGGATGTGTACAAG  900
901   ATTGGGGGCATTGGGACCGTGCCTGTGGGCCGAGTGGAGACCGGTATCCTCCGGCCTGGT  960
961   ATGGTGGTGACCTTTGCGCCAGTCAACATCACCACAGAGGTGAAGTCTGTGGAAATGCAC  1020
1021  CATGAGGCACTCAGCGAGGCCCTGCCTGGTGACAATGTCGGGTTCAATGTGAAGAATGTG  1080
1081  TCTGTTAAGGATATTCGCCGGGGCAATGTCTGCGGGGACAGCAAAGCTGACCCCCCTCAG  1140
1141  GAGGCTGCCCAGTTCACCTCTCAGGTTATCATCCTGAACCACCCTGGGCAAATCAGCGCT  1200
1201  GGCTACTCGCCAGTCATAGACTGTCAGACAGCCCACATTGCCTGCAAGTTTGCCGAGCTA  1260
1261  AAGGAGAAGATTGAACGTCGCTCTGGCAAGAAGCTGGAGGACAATCCCAAGTCCCTGAAG  1320
1321  TCTGGCGATGCAGCAATTGTAGAGATGGTCCCAGGAAAACCAATGTGTGTGGAGAGCTTC  1380
1381  TCACAGTACCCACCCCTCGGCCGCTTCGCAGTACGCGACATGCGGCAGACAGTGGCCGTG  1440
1441  GGCGTCATCAAGAACGTGGAGAAGAAGAGCGGCGGCGCCGGCAAGGTCACAAAATCCGCA  1500
1501  CAGAAGGCGCAGAAAGCGGGCAAGTGAACCGGCGCCCGGCGAGGACCTCAACGAGCCCGA  1560
1561  CGCGCCCCCCAGGCCCCCGCCGCACTGGCCCCGTGCGGTCCCCGCCGCAGCGCGCCCCTC  1620
1621  TGCCCCGACCCCCTGCCAGGCTCATGTCTGCACCTCCGCTTGTAAGAGGCTCTACGTCAG  1680
1681  CGACTGGATGCTCGCCATCAAAGTCCAGTGGAAATTCTTGAAGAGGAAAAGCGCCCGCCC  1740
1741  CAGTTTCGCCAGCGCTCACCACGCTCAAGTGCCCGTTCTCCCAATAAACTGAGCGACCCC  1800
1801  AAAAAAAA  1808
```

Figure 3. Nucleotide sequence of the pHH-S1 insert. The putative stop codon and the poly(A) addition signal are underlined. Notice the GC-rich sequence after the stop codon that is characteristic of the S1 mRNA.

```
1    CTACAAATGCGGAGGTATTGACAAAAGGACCATTGAGAAGTTCGAGAAGGAGGCGGCTGA 60
61   GATGGGCAAGGGATCCTTCAAGTATGCCTGGGTGCTGGACAAGCTGAAGGCGGAGCGTGA 120
121  GCGCGGCATCACCATCGACATCTCCCTCTGGAAGTTCGAGACCACCAAGTACTACATCAC 180
181  CATCATCGATGCCCCGGGCCACCGCGACTTCATCAAGAACATGATCACGGGTACATCCCA 240
241  GGCGGACTGCGCAGTGCTGATCGTGGCGGCGGGCGTGGGCGAGTTCGAGGCGGGCATCTC 300
301  CAAGAATGGGCAGACGCGGGAGCATGCCCTGCTGGCCTACACGCTGGGTGTGAAGCAGCT 360
361  CATCGTGGGCGTGAACAAAATGGACTCCACAGAGCCGGCCTACAGCGAGAAGCGCTACGA 420
421  CGAGATCGTCAAGGAAGTCAGCGCCTACATCAAGAAGATCGGCTACAACCCGGCCACCGT 480
481  GCCTTTTGTGCCCATCTCCGGCTGGCACGGCGACAACATGCTGGAGCCCTCCCCCAACAT 540
541  GCCGTGGTTCAAGGGCTGGAAGGTGGAGCGTAAGGAGGGCAACGCAAGCGGCGTGTCCCT 600
601  GCTGGAGGCCCTGGACACCATCCTGCCCCCCACGCGCCCCACGGACAAGCCCCTGCGCCT 660
661  GCCGCTGCAGGACGTGTACAAGATTGGCGGCATTGGCACGGTGCCCGTGGGCCGGGTGGA 720
721  GACCGGCATCCTGCGGCCGGGCATGGTGGTGACCTTTGCGCCAGTGAACATCACCACTGA 780
781  GGTGAAGTCAGTGGAGATGCACCACGAGGCTCTGAGCGAAGCTCTGCCCGGCGACAACGT 840
841  CGGCTTCAATGTGAAGAACGTGTCGGTGAAGGACATCCGGCGGGGCAACGTGTGTGGGGA 900
901  CAGCAAGTCTGACCCGCCGCAGGAGGCTGCTCAGTTCACCTCCCAGTCATCATCCTGAAC 960
961  CACCCGGGGCAGATTAGCGCCGGCTACTCCCCGGTCATCGACTGCCACACAGCCCACATC 1020
1021 GCCTGCAAGTTTGCGGAGCTGAAGGAGAAGATTGACCGGCGCTCTGGCAAGAAGCTGGAG 1080
1081 GACAACCCCAAGTCCCTGAAGTCTGGAGACGCGGCCATCGTGGAGATGGTGCCGGGAAAG 1140
1141 CCCATGTGTGTGGAGAGCTTCTCCCAGTACCCGCCTCGGCCGCTTCGCCGTGCCCGACAT 1200
1201 GAGGCAGACGGTGGCCGTAGGCGTCATCAAGAACGTGGAGAAGAAGAGCGGCGGCGCCGG 1260
1261 CAAGGTCACCAAGTCGGCGCAGAAGGCGCAGAAGGCGGGCAAGTGAAGCGCGGGCGCCCG 1320
1321 CGGCGCCGACCCTCCCCGCGGCGCCGCGCTCCGAACCCCGGCCCGGCCCCCGCCCCGCCC 1380
1381 CCGCCCCGTCGCCGCTCCGGCGCCCCGCACCCCCGCCAGGGGCAGTCTGCACCTCCGCTT 1440
1441 GCCAGAGGCCCTCGGTCAGCGACTGGATGCTCGCCATCAAGGTCAGTGGAAGTTCAAGAG 1500
1501 GAAAGGCGCCCCCGCCCCAGGCTTCCGCGCCCAGCGCTCGCCACGCTCAGTGCCCGTTTT 1560
1561 ACCAATAAACTGAGCGACCCCAAAA 1585
```

Figure 4. Comparison between the mouse and human S1 deduced amino acid sequence, with that of rat S1 and human EF-1α. The amino acid sequences are predicted from the cloned cDNAs of mouse (MS1) and human (HS1) S1, rat (RS1) S1 (7, 8), and human (HEF) EF-1α (10, 11). Dashed lines indicate identity, whereas dots are introduced to maximize the alignments. Note that the only difference between rat and human S1 is a conservative substitution (A->S) at rat S1 residue 331.

```
MS1:  MGKEKTHINI  VVIGHVDSGK  STTTGHLIYK  CHHIDKRTIE  KFEKEAAEMG  50
HS1:  ..........  ..........  .........YK  CGGIDKRTIE  KFEKEAAEMG  22
RS1:  MGKEKTHINI  VVIGHVDSGK  STTTGHLI--  ----------  ----------  50
HEF:  MGKEKTHINI  VVIGHVDSGK  STTTGHLI--  ----------  ----------  50

MS1:  KGSFKYAWVL  DKLKAERERG  ITIDISLWKF  ETTKYYITII  DAPGHRDFIK  100
HS1:  KGSFKYAWVL  DKLKAERERG  ITIDISLWKF  ETTKYYITII  DAPGHRDFIK  72
RS1:  ----------  ----------  ----------  ----------  ----------  100
HEF:  ----------  ----------  ----------  --S---V---  ----------  100

MS1:  NMITGTSQAD  CAVLIVAAGV  GEFEAGISKN  GQTREHALLA  YTLGVKQLIV  150
HS1:  NMITGTSQAD  CAVLIVAAGV  GEFEAGISKN  GQTREHALLA  YTLGVKQLIV  102
RS1:  ----------  ----------  ----------  ----------  ----------  150
HEF:  ----------  ----------  ----------  ----------  ----------  150

MS1:  GVNKMDSTEP  AYSEKRYDEI  VKEVSAYIKK  IGYNPATVPF  VPISGWHGDN  200
HS1:  GVNKMDSTEP  AYSEKRYDEI  VKEVSAYIKK  IGYNPATVPF  VPISGWHGDN  152
RS1:  ----------  ----------  ----------  ----------  ----------  200
HEF:  ----------  P--Q---E--  -----T----  -----D--A-  ------N---  200

MS1:  MLEPSPNMPW  FKGWKVERKE  GNASGVSLLE  ALDTILPPTR  PTDKPLRLPL  250
HS1:  MLEPSPNMPW  FKGWKVERKE  GNASGVSLLE  ALDTILPPTR  PTDKPLRLPL  202
RS1:  ----------  ----------  ----------  ----------  ----------  250
HEF:  -----A----  ------T--D  -----TT---  ---C------  ----------  250

MS1:  QDVYKIGGIG  TVPVGRVETG  ILRPGMVVTF  APVNITTEVK  SVEMHHEALE  300
HS1:  QDVYKIGGIG  TVPVGRVETG  ILRPGMVVTF  APVNITTEVK  SVEMHHEALE  252
RS1:  ----------  ----------  ----------  ----------  ----------  300
HEF:  ----------  ----------  V-K-------  ----V-----  ----------  300

MS1:  ALPGDNVGF   NVKNVSVKDI  RRGNVCGDSK  ADPPQEAAQF  TSQVIILNHP  350
HS1:  ALPGDNVGF   NVKNVSVKDI  RRGNVCGDSK  SDPPQEAAQF  TSQVIILNHP  302
RS1:  ----------  ----------  ----------  ----------  ----------  350
HEF:  ----------  ---------V  -----A----  N---M---G-  -A--------  350

MS1:  GQISAGYSPV  IDCHTAHIAC  KFAELKEKID  RRSGKKLEDN  PKSLKSGDAA  400
HS1:  GQISAGYSPV  IDCHTAHIAC  KFAELKEKID  RRSGKKLEDN  PKSLKSGDAA  352
RS1:  ----------  ----------  ----------  ----------  ----------  400
HEF:  -------A--  ----------  ---------G  ----------  --F-------  400

MS1:  IVEMVPGKPM  CVESFSQYPP  LGCHTAHIAC  QTVAVGVIKN  VEKKSGGAGK  450
HS1:  IVEMVPGKPM  CVESFSQYPP  LGCHTAHIAC  QTVAVGVIKN  VEKKSGGAGK  402
RS1:  ----------  ----------  ----------  ----------  ----------  450
HEF:  --D-------  ------D---  ----------  ---------A  -D---AA---  450

MS1:  VTKSAQKAQK  AGK   463
HS1:  VTKSAQKAQK  AGK   435
RS1:  ----------  ---   463
HEF:  ----------  -.-   462
```

Table 1

Sequence comparison between different species EF-1αs, and mouse and human S1, with rat S1

Species	Sequence similarity (%)			Putative functional domains		Modified residues[2]
	5' UTR	3' UTR	Amino. acids	G-binding	AA-tRNA	CH3, GPEA, PO_4^-
Rat EF-1α	<25[1]	<25	92.6	Conserved	Conserved	All conserved
Mouse EF-1α	<25	<25	92.8	Conserved	Conserved	All conserved
Human EF-1α	<25	<25	91.8	Conserved	Conserved	All conserved
Mouse S1	>90	>90	100	Conserved	Conserved	All conserved
Human S1	N.D.[3]	>90	99.8	Conserved	Conserved	All conserved

[1] <25; no significant similarity

[2] Known post-translation modified residues

[3] Not determined

Discussion

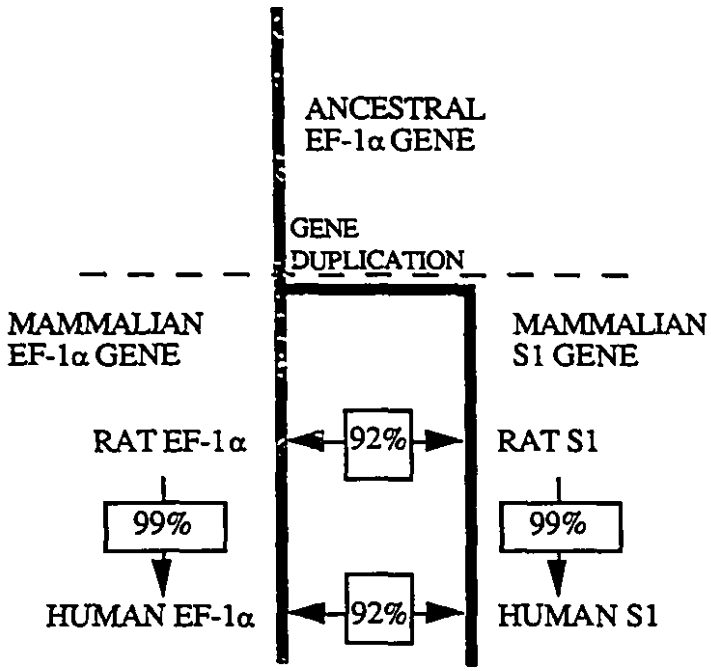
We report here the isolation of cDNAs from mouse and human brain cDNA library. We argue that pMB-S1 and pHH-S1 are the mouse and human homologue of rat S1 because of: (1) the high nucleotide similarity in the 5' and 3' UTR and (2) the total amino acid conservation. The cloning of mouse and human S1 is consistent with our hypothesis that S1 is present in all mammals, and that it is the second member of the mammalian EF-1α gene family.

The nucleotide sequence of pHH-S1 is totally similar to that of EF-1α2 reported by another group (16) indicating that human S1 and human EF-1α2 are the same genes. The same authors have shown that EF-1α2 tissue expression is limited to human brain, heart, and muscle which is consistant with our in vivo observation of tissue-specific distribution of S1 mRNA in rat and mouse. The chromosomal gene and a cDNA for human EF-1α have already been characterized, along with several retropseudogenes (10, 11). Madsen et al. (1990, 17) have suggested that there is only one active EF-1α gene copy per genome in man. They arrived at this conclusion after analyzing several EF-1α cDNAs isolated from liver, fibroblasts, and lymphoid libraries that contained the same nucleotide sequences. Yet they reported the presence of another chromosomal EF-1α-like gene that contained introns, but argued that this constituted an inactive gene. We suggest that this second gene codes for human S1 (EF-1α2). The reason that the S1 cDNA was not isolated by Madison et al. (1990, 17) is probably because they screened libraries that were constructed from RNA isolated from S1-negative cells and tissues. We also screened a WI-38 fibroblast library with the pS1-7 probe and did not obtain any positive clones (Fig. 1; annex).

Mammalian species' EF-1α mRNA 3' UTRs are highly conserved and somewhat similar to that of Xenopus laevis EF-1αS, the somatic form of EF-1α (8, 18). S1 mRNA 3'

UTR lacks homology with any EF-1α from other species. This lack of homology suggests that the S1 gene has emerged late in evolution, and is specific to mammalian species. It also suggests that it has acquired distinct transcriptional specificity by recombination of the promoter sequence as well as the 3' UTR during evolution from an ancestral EF-1α gene. It is interesting that rat pS1 residues, which differ from EF-1α, have been totally conserved in mouse and man pS1. The conservation of the changed residue between S1 and EF-1α suggests that these changes have led to distinct functions for S1, and have been submitted to strong evolutionary pressure. If the amino acid differences between rat S1 and EF-1α had not led to a change in function, they would not have been so highly conserved in mouse and man. We argue that, while S1 probably binds GTP and aminoacyl-tRNA, it does not function exactly as EF-1α. The results presented here are consistent with our hypothesis, and suggest that S1 functions distinctively from EF-1α in regulating certain aspects of the elongation step of protein synthesis in cells that are long-lived, and have permanently withdrawn from the cell cycle such as neurons and myocytes.

Figure 5. Schematic diagram depicting a model of the evolution of the S1 gene.
In this model, the S1 gene has evolved from an ancestral EF-1α gene by gene duplication. The pS1 amino acid residues that differ from mammalian EF-1α have been totally conserved in mammals. Because there is no sequence similarity between the 3' UTR of the S1 mRNA and other nonmammalian EF-1αs, we suggest that the S1 gene is specific to mammalian species.


ANCESTRAL
EF-1α GENE
GENE
DUPLICATION
MAMMALIAN
EF-1α GENE
MAMMALIAN
S1 GENE
RAT EF-1α
92%
RAT S1
99%
99%
HUMAN EF-1α
92%
HUMAN S1

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CHAPTER 6

GENERAL DISCUSSION

The results presented in this thesis have already been extensively discussed in their respective chapters. Chapter 6 includes a recapitulation of the data (Table 1) and a résumé of chapters 2 to 5 discussions. It also contains a section that is devoted to hypotheses and speculations as to S1 cellular function. Also included are examples of future experiments that are deemed necessary to test the mentioned hypotheses.

1. Mammalian EF-1α gene family

The cloning of the S1 cDNA (Ann et al. 1991) indicated that rat contained at least two members of the EF-1α gene family. We directly demonstrated that S1 was not a rat homologue of mammalian EF-1α, when we reported the isolation of a partial rat EF-1α cDNA (REF3) (Ann et al., 1992). We argued that REF3 codes for rat EF-1α because this clone contains a 3' UTR highly similar to that of mouse, rabbit, and human EF-1α. The divergence in the 3' UTR between rat S1 and rat EF-1α provided an efficient means to prepare specific 3' UTR cRNA probes by in vitro transcription, which were the principal tools used for the presented work.

S1 is not a rat-specific gene, as shown by RNase protection and Northern analysis in mouse and by cloning of the mouse and human S1 cDNAs. The identification of S1 in these species was facilitated by the fact that the S1 3' UTR is highly conserved in mammals, a characteristic also noticed in EF-1α mRNA. On the basis of the sequence similarity between S1 and EF-1α, we conclude that S1 is the second member of the mammalian EF-1α gene family. One important aspect of the mammalian EF-1α gene family is the presence of several retropseudogenes (Madsen et al., 1990). Our results suggest that the retropseudogenes in rat have originated from the EF-1α mRNA, and not S1 mRNA, which is present in only one copy in the rat genome. How many active EF-1α genes are there in mammals? It is possible that early embryos express a specific EF-1α gene, as in

Table 1

Comparison between members of the EF-1α gene family of different species

Results in bold were obtained by the author of this thesis

Species	Genes	Sequence similarity (%)		Differential Expression			Function
(# genes)	Name	3' UTR[1]	A.acids	Embryonic	Adult	Aging	
S. Cerevisiae (2)	TEF1	<25	100	No difference	No difference	N.D.[2]	In peptide elongation
	TEF2			No difference	No difference		In peptide elongation
M. racemous (3)	TEF1	<25	100	Methylation level	High expression	N.D.	N.D.
	TEF2			Idem	High expression		
	TEF3			Idem	Low expression		
D. melanogaster (2)	F1	<25	92	All tissues	All tissues	Decrease	N.D.
	F2			Pupal stage only	No expression	N.D.	
X. Laevis (3)	EF-1αO	<25	>63	Until neurulation	Germ cell lines-specific	N.D.	In peptide elongation
	EF-1αS			After blastulation	All tissues		Binds GTP, a.a.tRNAs
	42Sp50			Until neurulation	No expression		In peptide elongation
Mouse (2)	EF-1α	**<25**	**92.7**	N.D.	**All cells, tissues**	???	N.D.
	S1[3]				**Brain, heart, muscle**	N.D.	N.D.
Human (2)	EF-1α	**<25**	**92.8**	N.D.	**All cells, tissues**	Fibroblasts	N.D.
	S1				**Brain, muscle**	N.D.	N.D.
Rat (2)	**EF-1α**	**<25**	**92.6**	**Tissue specific manner**	**All cells, tissues, different levels**	**No change mRNA**	In peptide elongation
	S1			**Post-terminal differentiation dependent**	**Brain (certain neurons), heart, muscle (myotubes)[4]**	**No change mRNA**	N.D.

[1] <25; no significant similarity.

[2] N.D.; not determined or does not apply

[3] Human S1 is 99.8% conserved with rat and mouse S1. 3' UTR also >90% conserved

[4] S1 and EF-1α expression overlaps in these cells and tissues, expression levels are similar.

Drosophilia and Xenopus (Hovemann et al., 1988; ,Walldorf et al., 1985; Dje et al., 1990; Mattaj et al., 1987; Viel et al., 1987; Bourne et al., 1991). Another possibility is that specific EF-1α genes are expressed in other adult tissues, such as S1 in brain, heart and muscle, and Xenopus EF-1αO in germ cells (Abdallah et al., 1991). However, it has been reported that the human genome contains only two EF-1α genes with introns, of which one codes for EF-1α (Uetsuki et al., 1989). It is probable that the second gene codes for human S1. Therefore, further genomic characterization will be necessary to reveal the presence or absence of other active members of the mammalian EF-1α gene family.

So why was S1 not previously identified? Indeed, the cloning of the S1 cDNA in rat came as a surprise, considering the amount of work published in the last 30 years on mammalian EF-1α. Yet, it can be easily understood. S1 and EF-1α have the same molecular weight (50kDa), pI (11.0), and transcript length (1.8-1.9 kb). These similar characteristics would have made, and still render, the identification of S1 difficult. Brechet et al. (1986) reported the isolation of calf brain EF-1α. In all their studies, they detected only one band by either one-or two-dimensional gel electrophoresis (Brechet et al., 1986; Brechet and Parmeggiani, 1986). Sanders et al. (1992) did not distinguish between S1 and EF-1α when they probed rat muscle total RNA with a full length human EF-1α cDNA. Finally, in the effort to isolate a second human EF-1α cDNA, Madsen et al. (1990) screened several cDNA libraries, and sequenced at least 15 independent clones with the same nucleotide sequence. They arrived at the conclusion that only one EF-1α gene was active in man. These results are explainable by the observation that the libraries were constructed from RNA isolated from liver, lymphoid cells, and several different fibroblast cell lines, sources that we now know to be S1-negative. Therefore, S1 would probably not have been identified if it was not for its furtuitous cloning during the attempt to isolate statin corresponding cDNA. Still, the full characterization of S1 is impeded by the fact that we cannot distinguish between EF-1α and pS1 at the protein level, until specific monoclonal

antibodies to these two peptides are obtained. Because of this limitation, we mainly focus our efforts on the characterization of S1 and EF-1α gene expression at the mRNA level, using the specific cRNAs that we constructed.

2. S1 and EF-1α expression

A) During development

Our work has shown that S1 gene expression is restricted to brain, heart, and muscle in rat, mouse, and man. In situ hybridization has failed to identify any S1-positive cells in liver, including the nonhepatocyte cells such as smooth muscle and endothelial cells that compose the venule tunique. We do not have any biochemical or molecular evidence to precisely identify S1-positive cell types in brain. Still, from the in situ hybridization work, we can deduce that: (1) S1 expression is restricted to certain cells in brain, and (2) S1 is expressed in Purkinje and motor neurons based, on the morphological structure and anatomical localization of some S1-positive cells. That S1 gene expression is limited to brain, heart, and muscle, added to the fact that certain neurons are S1-positive led us to hypothesize that S1 is a terminal differentiation-specific EF-1α-like protein. This hypothesis was strengthened by the results obtained in the developmental studies. S1 expression is a late event during brain, heart, and muscle development, and its mRNA accumulates at a time when cells are permanently leaving the cell cycle (Olson, 1993; Sassoon, 1993). That S1 mRNA can only be detected after the formation of myotubes in culture suggests that this gene is part of a postdifferentiation program (Yaffe and Saxel, 1977). Our hypothesis is in agreement with the suggestion of Baltimore and Blau (1991) that any cellular differentiation state is an active phenomenon, requiring continuous regulation (see also Blau, 1992). We propose that S1 functions as part of a general mechanism to keep neurons, myocytes, and cardiomyocytes in a state of nonproliferation.

Along the same line of investigation, we have shown that EF-1α mRNA can be detected in every tissue and cell type that we have studied in rat. While this seems obvious, no direct demonstration of EF-1α mRNA tissue and cellular distribution has ever been reported in mammals. EF-1α expression is also highly modulated during development, and its mRNA does not accumulate to the same levels in brain, heart, muscle and liver. The general decrease of EF-1α mRNA level during brain, heart, and muscle development may reflect the terminal differentiation process. Actually, EF-1α levels decrease late during heart and muscle development, suggesting that EF-1α gene expression is repressed after the formation of post-mitotic muscle cells (Olson, 1993; Sassoon, 1993). This phenomenon is less appreciable in brain, but this may reflect the high cellular heterogeneity of this organ. Furthermore, proliferating cardiomyoblasts, neuroblasts and myoblasts probably have a higher need for protein synthesis than do their nonproliferating counterparts, and this may be why EF-1α mRNA levels are higher during early development.

One interesting aspect of the developmental studies is that EF-1α mRNA down-regulation is preceded by the up-regulation of S1 mRNA. Furthermore, EF-1α mRNA levels do not change during liver development. This may be a simple coincidence, as we have not investigated EF-1α developmental expression in other S1-negative tissues such as kidney or spleen. Still, the accumulation of either S1 mRNA or protein could be directly responsible for the downregulation of EF-1α gene expression. Such cross-talk between EF-TuA and EF-TuB genes has been reported in procaryotes (van der Meide et al., 1983; van Delft and Boosch, 1988; van Delft et al., 1988). The fact that EF-1α mRNA levels do not decrease during myogenesis in culture can be explained by the observation that S1 mRNA does not accumulate to high levels. Therefore, if S1 mRNA accumulated to high level in L8 myotubes, we would see a decrease in EF-1α transcript. This hypothesis is testable by either high efficiency transient transfection of the S1 cDNA, or the establishment of stable transfected S1 cell lines.

B) During aging

There are several reports implicating EF-1α in aging. A decline in EF-1α mRNA levels, protein levels, and activity has been reported for several species (Cavallius et al., 1986, 1989; Rattan et al., 1986; Webster and Webster, 1983, 1884; Webster, 1985; Castaneda et al., 1986). However, no accurate techniques have been employed to obtain these data. We have shown, by Northern analysis and RNase protection assays using specific probes, that there are no differences in S1 and EF-1α mRNA levels during the normal mammalian adult life span. The modulation of the expression of both genes only occurs early during the life of this organism, and we think that this phenomenon is related to the different need for protein synthesis during development. If members of the EF-1α gene family are implicated in mammalian aging, such changes are not regulated at the mRNA level. Our results do not exclude a possible drop in EF-1α or S1 activity due to translation or post-translation regulatory mechanisms.

3. S1 cellular function

A) Sequence analysis

This is the most intriguing question yet to be solved. Why do terminally differentiated cells already expressing EF-1α need S1? We have inferred a role for S1 in the elongation step of translation, because of its high sequence similarity to EF-1α. In fact, there are three arguments suggesting that S1 plays a role in elongation; the first consists of the total conservation of the functional domains, such as the GTP and the tRNA binding domains, in the primary amino acid sequence of S1 (Dever et al., 1987; Valencia et al., 1991; van Damme et al., 1992; Kinzy et al., 1992). All the amino acids that are known to be post-translated in EF-1α are also present in S1 (Dever et al., 1989; Toledo and Jerez, 1989; Whiteheart et al., 1989). The sequence conservation of the putative functional

domains strongly suggests that S1 behaves in a similar fashion to EF-1α. The second argument is that all species contain several EF-1α-like genes that code for proteins implicated in elongation. Therefore, it would not be surprising if mammals also contain such an EF-1α like gene, for which S1 is the best candidate. The third argument is that S1 mRNA accumulates to levels comparable to those of EF-1α. While we do not know to which level pS1 accumulates, it does indicate that this protein is a major constituent of certain cell types. The high abundance of S1 also suggests functional similarity to EF-1α.

These three arguments do not necessarily imply functional redundancy, as seems to be the case in lower species (Linz et al., 1986; Cottrelle et al., 1985; Hughes et al., 1990). The EF-1α protein has been almost totally conserved in mammals. This fact suggests that any mutations occurring in the primary amino acid sequence of EF-1α would led to functional aberrations, and eventually to the death of the organism. S1 does have 35 different amino acid compared with EF-1α. These are not random events, as the substitutions have been conserved from rat to man. If the amino acid modifications had not resulted in the modification of function, they would not have been conserved between the two species, and pS1 would not have been so highly conserved between rat and man. Hence, our data implies that terminally differentiated cells contain a specific translation elongation machinery in which S1 is probably implicated. This is, to our knowledge, the first report on the identification of an altered translation factor in any cell type in mammals.

What is puzzling is that in situ hybridization, and myogenic cultures, suggest that S1-positive cells also contain EF-1α mRNA. If EF-1α mRNA were not found, it would be reasonable to speculate that S1 behaves as EF-1α, specific for the protein synthesis machinery of these cells. One possibility is that EF-1α mRNA is not translated in these cells. This is not an idle speculation, as there is one report that indicates translation repression of the EF-1α mRNA in nonproliferating murine fibroblasts (Slobin and Rao,

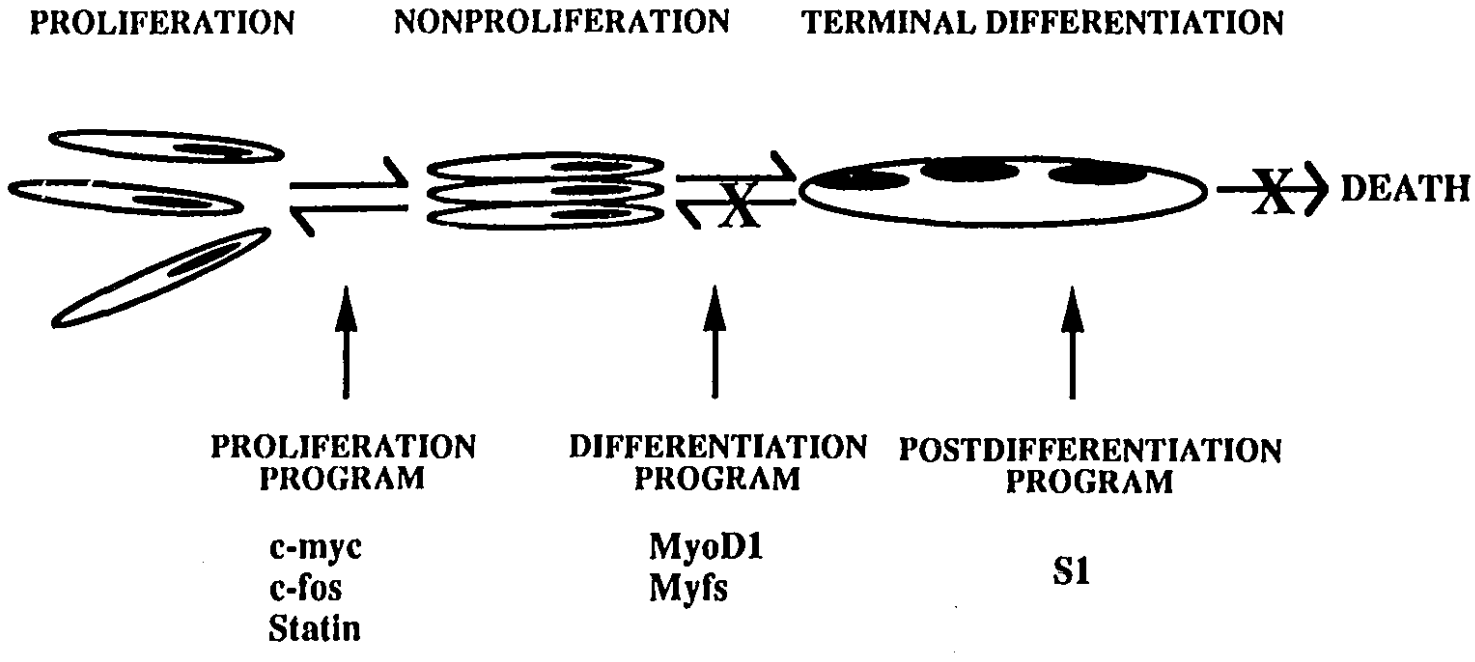
1993). The trans-acting factor, implicated in this repression, could accumulate to high levels in permanently growth arrested cells. The decrease in EF-1α mRNA detected during development, added to a putative translation repression, may be mechanisms to eliminate, or at least reduce, the amount of EF-1α protein. Here, the differences in the UTRs between S1 and EF-1α may be important in permitting S1 mRNA translation and pS1 accumulation in such cells. Such a regulatory role for the 3' UTR of myogenic specific genes has already been reported (Rastinejad and Blau, 1993).

B) Speculation

Cellular proliferation provides tissues not only with regeneration capacities, but also with a selective cellular turnover which allows the replacement of damaged or aged cells. The possibilities of cellular turnover and regeneration represent essential functional systems to keep individual organisms viable for many years or decades (Medvedeva and Medvedeva, 1991). Still, there are some forms of cellular differentiation that serve functions which are incompatible with cellular replacement or regeneration, because the loss of cells also means the loss of essential information necessary for survival. Neurons are the most obvious examples of such irreplaceable cells, even in long-lived organisms (Crespo et al., 1986). The maximum life span of such cells is the same as the maximum life span of the whole organisms. The evolution that created cellular proliferation to keep functional integrity in liver, also created some still unknown molecular mechanisms for maintaining the integrity of highly differentiated cells throughout the life span of individual organisms. As mentioned above, Blau and Baltimore (1991) proposed a model by which differentiation is an active process requiring continual regulation. We hypothesize that S1 is part of a program to keep neurons, cardiomyocytes and myocytes nonproliferating as well as long-lived in mammals (Fig. 1). We suggest that these cells contain a specific translation machinery

Figure 1. Schematic diagram of S1 putative cellular function

Myogenic terminal differentiation is a well characterized system. To form myotubes, myoblasts must first stop proliferating (depicted as nonproliferation program). This phenomenon is reversible, as myoblasts are capable of reentering the cell cycle. Once myoblasts have stop proliferating, they can readly fuse to form multinucleated myotubes. This step of differentiation is thought to be regulated by myogenic determination factors such as MyoD1, Myfs, etc. Once fused, myotubes are incapable of reentering the cell cycle, a cellular state known as terminal differentiation. Neurons and cardiomyocytes resemble myotubes, as they too are incapable of reentering the cell cycle. Terminal differentiation is not a state specific to myocytes and neurons, as several other cells, such as keratinocytes, are incapable of further cellular division. However, these cells have a relatively short life span, whereas myocytes' and neurons' life span is the same as the maximum life span of the whole organisms. We hypothesize that myocytes and neurons, once totally differentiated, express a specific post-differentiation program to prevent both the reentry into the cell cycle, as well as cellular death, during the life span of the organism. The tissue- and cell type-specific expression of S1, as well as the fact that it is express after differentiation, make S1 a good candidate for such a program.


PROLIFERATION
NONPROLIFERATION
TERMINAL DIFFERENTIATION
DEATH
PROLIFERATION PROGRAM
c-myc
c-fos
Statin
DIFFERENTIATION PROGRAM
MyoD1
Myfs
POSTDIFFERENTIATION PROGRAM
S1

of which S1 is a part. How S1 functions to keep cells nonproliferative and long-lived is still unclear. But, our hypothesis is strengthened by the fact that transgenic Drosophila which express an extra copy of EF-1α live longer (Shepherd et al., 1989). How the overexpression of EF-1α results in increase life-span is not known. The only explanation suggested so far is that high level of EF-1α enhances translation fidelity, as reported experimentally (Song et al., 1989) and predicted by computer analysis (Pingoud et al., 1990). Hence, S1 may be part of a mechanism necessary to prevent the accumulation of aberrant proteins that would be detrimential to cells having no regenerating capacity. Such mechanims would be necessary to maintain cellular integrity for long-lived cells and organisms.

4. Future prospects

The data presented in this thesis has provided information on the differential expression of S1 and EF-1α. We have demonstrated that S1 expression is subsequent to the terminal differentiation process, and that S1 mRNA can be detected in certain cell types such as neurons and myocytes in mammalian species. This information can now be used to investigate S1 cellular function.

A) Regulation analysis

Our developmental studies have suggested that the S1 gene expression is post-terminal differentiation dependent. One line of investigation would be to dissect the S10 promoter to find elements and identify trans-acting factors that confer such an expression profile. The principal difficulty would consist in selecting an adequate cell system for these studies. One problem that we have encountered during our work was to identify a proper cell line. S1 is expressed in established myogenic cell lines, but its mRNA accumulates to a fraction of the levels observed in vivo. It would be more appropriate to carry on with

regulatory work on S1 using primary cultures of cells isolated from S1-positive tissues. While the isolation and separation of cardiomyocytes from noncardiomyocytes is not trivial, it should serve as an adequate system for these studies. However, the verification that cells are still expressing S1 after such procedures would be imperative. The following experiments would follow well-established protocols, such as transient transfection of S10 promoter-CAT constructs, DNA foot-printing and gel retardation assays. This work should reveal new regulatory elements and trans-acting factors implicated in gene expression specific to the post-terminal differentiation process.

B) Functional analysis

The most obvious question that must be eventually answered is what is S1's relation to EF-1α? We have suggested two hypotheses: (1) S1 functions in a similar fashion to EF-1α, but with certain distinctive characteristics specific to terminally differentiated cells, and (2) S1 is part of a postdifferentiation mechanism to keep neurons and myocytes long-lived in mammals. Both hypotheses are readily testable and will be treated separately:

(1) The easiest way to test this hypothesis is to produce an in vitro system such as the ones originally used to identify EF-1α activity. These systems contain initiated ribosomes, EF-1 complex with EF-1α depleted by heat inactivation, EF-2, [^{14}C]Phe-tRNA, poly(U) RNA and appropriate buffers. pS1 is obtained by in vitro translation and added to the assay mixture. The amount of [^{14}C]Phe incorporated into nascent polypeptides is measured by TCA precipitation. The major downfall with this experiment is that both positive and negative results are questionable. In vitro translated S1 does not have its normal complement of post-translation modifications. Moreover, the translation components are not isolated from cells that express S1. It could be argued that pS1 is not in its normal cellular environment. Therefore, a negative result is easily explainable by the notion that S1 cannot interact with components of cells other then neurons and myocytes.

Furthermore, a positive result can be explained by the idea that pS1 would function as an EF-1α in S1-negative cells, and that it is not express in these cells to avoid functional redundancy. Hence, such experiments, while the most obvious, would not give clear results on pS1 cellular function.

We suggest other avenues of investigation. The first avenue consists of comparing the immunoprecipitation profile of S1 to that of EF-1α from S1-positive cells using S1 and EF-1α specific antibodies. This experiment should reveal whether S1 can bind to EF-1βγ to form the EF-1 complex. Specific antibodies could also reveal if S1 binds to all the aminoacyl-tRNAs, or prefers its own pool in neurons and myocytes. Transfection experiments of the S1 cDNA into S1-negative cells should tell us whether S1 can form an EF-1 complex and bind aminoacyl-tRNA in cells where it is normally not expressed. One possibility is that S1 competes with EF-1α for peptide elongation factors. Transfection experiments followed by the proposed analysis and measurement of elongation parameters such as elongation rate should provide information on S1 function. Finally, a more demanding experiment would be to purify all the peptide elongation factors, such as those enumerated before, from S1-positive cells. S1, and EF-1α, could be purified from these cells and added to the extracts to measure peptide elongation activity. These experiments should convincingly evaluate S1 function as an EF-1α-like protein in S1-positive cells.

The proposed experiments are all feasible, but only with specific antibodies to pS1 and EF-1α. In the past four years, efforts have been made to produce such antibodies. Strategies of injecting rabbits or mice with full-length peptides or oligopeptides to specific regions have failed. Other groups have also failed in their attempt to produce antibodies to EF-1α. EF-1α and probably S1 are very abundant proteins that are totally conserved in mammals. It is possible that such characteristics render immunogenic response to these proteins impossible. We will eventually need these tools to perform the listed experiments

and fully characterize S1 function in neurons and myocytes. Therefore, we are limited in our experiments by the fact that the only tools presently available to distinguish S1 from EF-1α are the described riboprobes.

(2) This hypothesis is complex and will be difficult to test. The detrimental effect of S1 absence probably spans over several months, thus excluding culture transfection assays. Transient transfections of the S1 cDNA in several cell lines has not led to any gross morphological or physiological changes in these cells. Therefore, we propose to test this hypothesis using transgenic mouse technology. Mice carrying S1 null mutations (S1-, S1-) are produced by homologous recombination of the S1 gene in stem cells following well-established protocols. The main downfall of null mutations is that embryos die early in development. This should not be the case with S1, because our developmental analysis indicates that S1 gene expression is a late event during embryogenesis. Hence, even if the null mutation causes death, we should have enough tissues to work with. However, our hypothesis predicts that null mutation of S1 should have a long-term detrimental effect on transgenic mice. It also predicts that increased cell death in brain, heart, and muscle would lead to neuro-, cardio- and myopathies early in transgenic mouse life compared to nontransgenic control; such pathologies are readily discernible. Another advantage is that primary cultures of normally S1-positive cells can be isolated from the null mutants. This would provide better systems to analyze S1 function in its in vivo environment. We could study cells in which S1 is normally present in high abundance, compared to primary cultures isolated from wildtype mice. Measurement of translation parameters, followed by the experiments proposed above in null mutants versus wildtype primary cultures, should yield important information on S1 function in terminally differentiated, long-lived cells.

5. Concluding remark

The identification of S1 has open a new field in the study of translation elongation in mammals. We have shown that S1 and EF-1α are two independent genes with different expression profiles. That S1 gene expression is dependent upon terminal differentiation, and limited to cells that are long-lived, suggests that these cells have evolved peptide elongation mechanisms specific for these characteristics. The challenge for the near future will be to elucidate these mechanisms.

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CONTRIBUTIONS TO ORIGINAL KNOWLEDGE*

This section is a mandatory requirement of Ph.D. theses submitted to the Faculty of Graduate Studies and Research, McGill University, Montréal.

Work presented in this thesis has focused on the molecular characterization of S1 and elongation factor-1 alpha in mammalian species. The following novel findings have been demonstrated, and are listed according to chapter.

CHAPTER 2: A partial cDNA (REF3) to rat elongation factor-1 alpha has been cloned, as mentioned in chapter 1. S1 and REF3 cDNAs have been subcloned into pGEM3z plasmid to produce gene-specific probes by in vitro transcription (cRNA) of the 3' UTRs of both mRNAs. Northern blots and RNase protection protocols have been developed to study the tissue distribution of S1 and EF-1α mRNAs. These studies revealed that S1 mRNA is only present in rat brain, heart, and muscle, whereas EF-1α mRNA has been observed in all tissues studied so far. The same data was obtained when Northern analysis was performed using other S1-specific probes targeted to the 5' UTR or the coding region of S1 mRNA. Furthermore, we expanded our observation on S1 in rat by demonstrating the presence of a S1 homologue and its specific tissue distribution in brain, heart, and muscle in mouse. Aging studies have shown that S1 and EF-1α mRNA levels stay unchanged during the normal life span of rat. These findings suggest that mammalian species contain a second EF-1α like gene, whose expression is limited to brain, heart, and muscle.

CHAPTER 3: A non-radioactive in situ hybridization protocol has been developed to confirm at the cellular levels our previous finding of S1 tissue-specific expression. To do so, a method to verify the production of full-length, digoxigenin-labeled cRNA was also reported. Using this method, we could not detect S1 mRNA in liver cells. In contrast, results showed that all liver cells are positive for EF-1α mRNA. In brain, S1 mRNA was detected in different regions such as the hippocampus, the cortex, the brain stem, and the cerebellum. The staining pattern suggested that certain neurons such as motor and Purkinje neurons were S1-positive in the latter two regions. In the hippocampus, all cells were EF-

1α positive. These results confirm our previous finding, and suggest that S1 expression is limited to certain neurons in brain, whereas EF-1α mRNA can be detected in all cells.

CHAPTER 4: The developmental regulation of S1 and EF-1α mRNA in rat was investigated. First, we showed by Southern analysis that the EF-1α retropseudogene phenomenon in the rat genome is attributable to the EF-1α mRNA and not S1, which is a gene present in one copy. S1 mRNA can only be detected late during myogenesis, cardiomyogenesis, and neurogenesis, and accumulates to attain a plateau early after birth. EF-1α mRNA levels are down-regulated in S1-positive tissues, and stay relatively steady in liver, during rat development. The S1 gene is highly expressed in S1-positive tissues, and its mRNA accumulates to levels similar to those of EF-1α. Furthermore, S1 expression is dependent upon the formation of myotubes during myogenesis in culture. We have also investigated S1 and EF-1α gene expression at the mRNA levels in several rat, mouse, and human cell lines. S1 mRNA can only be detected in skeletal muscle cell lines, whereas smooth muscle, skin, normal fibroblasts, and transformed fibroblasts were all S1-negative. EF-1α mRNA accumulates to high levels independently of culture conditions. These results suggest that S1 is a unique, highly expressed gene, the expression of which is specific to the terminal differentiation-dependent process during rat development in vivo and in culture.

CHAPTER 5: The mouse (pMB-S1) and human (pHH-S1-1) homologues of rat S1 were cloned from a mouse brain and a human hippocampus cDNA library, using rat pS1-7 as the specific probe. The nucleotide sequence of pMB-S1 pHH-S1-1 indicates that the coding region and the UTRs of mouse and human S1 mRNA are highly homologous to those of rat S1. The predicted amino acid sequence from pMB-S1 and pHH-S1-1 is totally similar, except for one conservative amino acid change, to that of rat S1. RNase protection analysis has indicated that S1 mRNA can also be detected in human skeletal muscle, but not in MG-63 fibroblasts, which is consistent with our previous findings. Furthermore, libraries

constructed from RNA extracted from human tissues and cells such as aorta, normal fibroblasts, and transformed fibroblasts were negative when screened with the pS1-7 probe. These findings show that the S1 gene and gene expression are highly conserved in mammalian species such as rat, mouse, and man.