A NOVEL APPROACH FOR THE STUDY OF SEQUENCES THAT CONTROL CYTOPLASMIC RNA STABILITY

by

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(1980)

Submitted in Partial Fulfillment
of the Requirements of the Degree of
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at the

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ABSTRACT

The major objectives of the research described herein were:

1) to evaluate the utility of a number of inducible promoters in NIH/3T3 cells and MEL cells for studies of RNA stability, and
2) to apply the c-fos induction system in NIH/3T3 cells to the analysis of determinants of RNA stability of human β-globin and human c-fos RNAs.

Three inducible promoters, Drosophila hsp70, human metallothionein II_A, and human c-fos have been studied. My results indicate that Drosophila hsp70 is inducible in NIH3T3 cells, but exhibits constitutive expression in MEL cells. Human metallothionein promoter showed limited inducibility at subtoxic $ZnCl_2$ concentrations in MEL cells, yet 3T3 cells were not viable for adequate periods to perform biologically meaningful RNA stability experiments. The use of human c-fos promoter driven constructs in NIH/3T3 cells has proven a satisfactory approach to the analysis of RNA stability. RNA transcribed under the control of c-fos promoter reaches a maximal level shortly after transcription is stimulated by refeeding of quiescent cells with media containing 15% calf serum. Under these experimental conditions RNA half-life can be determined by directly measuring levels of the RNA species transcribed from the introduced DNA construct by Northern blotting of RNA samples prepared from various times following induction. Half-lives were calculated by the best-fit line of the log of normalized absorbances of these Northern blots plotted against time of chase of the sample. The following results have been obtained: human c-fos exhibited a half-life of 6.6 minutes, human β-globin exhibited a half-life of 17.5 hours. The large difference in half-lives between c-fos and β-globin made quantitative analysis of chimeric constructs between these RNAs of particular interest. Analysis of chimeric constructs indicates that determinants of RNA stability reside in at least two parts of the RNA molecule. Replacement of the 3' end of fos with the 3' end of globin stabilized the fos RNA 4-fold, yet the reciprocal chimera destabilized the globin message 30-fold. Replacement of the 5' end of fos with the 5' untranslated region of globin had no apparent effect on the stability of the fos RNA, yet the reciprocal chimera destabilized the globin message 2.5-fold. All RNAs tested have been found to be associated with ribosomes. This thesis provides an approach to the precise identification of RNA sequences or structures which control RNA stability.

Thesis supervisor: Dr. David E. Housman
Title: Professor of Biology

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Table of Contents

Title Page		1
Abstract		2
Acknowledgments		3
Table of Contents		4
I	Introduction	6
	A Review of the Literature	7
	1 Steady-state	8
	2 Perturbations	11
	3 Hormones	12
	4 Development and Differentiation	14
	5 Growth Control During the Cell Cycle	16
	6 Mutants	17
	7 A Technical Aside	19
	B General Features that Affect Stability	21
	1 Growth Rate and State of Confluence	21
	2 Translation	22
	3 Polyadenylation	24
	4 5' Terminal Cap	25
	5 Length of the RNA	26
	C Mechanisms of Degradation	27
	D Experimental Approaches for the Study of RNA Stability	30
	1 ^{3}H-Uridine Pulse/Chase	30
	2 Kinetics of Approach to Equilibrium	31
	3 Use of Drugs to Arrest Transcription	32
	E Our Approach	34
	1 Human c-*fos* Promoter	35
	2 Other Promoters Considered	35
II	Use of the Heat Shock Promoter	38
	A Introduction	39
	B Results	41
	1 CAT Activity in 3T3 Cells	41
	2 *myc* RNA in 3T3 Cells	42
	3 CAT Activity and CAT RNA in MEL Cells	49
	C Discussion	55
III	Use of the Metallothionein Promoter	66
	A Introduction	67
	B Results	69
	C Discussion	74
IV	Use of the Fos Promoter	82
	A Introduction	83
	B Results	84
	1 Preparation of Plasmids	84
	2 Determination of RNA Half-Lives	109
	3 State of Confluence	121
	4 Growth of Cells	121
	5 Translation	125
	C Discussion	130
	1 Half-Lives of Chimeric RNAs	130
	2 Mechanism	132
	3 Effects of Cell Confluence	133
	4 Translation	133
	5 Is Half-Life Dependent Upon Cellular Conditions?	135
	6 Conclusions	137

V Conclusions 163
VI Appendix 167
A Densitometry 168
B Quantitative Analysis 169
C Statistical Analysis 177
VII Materials and Methods 178
VIII Literature cited 190

Chapter I

Introduction

The eukaryotic cell has developed an elegant and complex system for controlling the expression of a given gene. On a gross level, a gene must be transcribed in order to be expressed. Finer tuning of steady state amounts of protein can be achieved by manipulation of the rates of degradation of nuclear RNA, nuclear RNA processing (splicing, capping, polyadenylation), transport across the nuclear envelope, post-translational modification of protein, degradation of mRNA, translational initiation, translational elongation, targetting of protein, degradation of protein, and others. Evidence in the current literature suggests, and in some cases proves, that mRNA stability plays a significant role in determining the level of expression in the steady state. A cell/system frequently needs to respond to a change in environment by rapidly changing the level of a given protein. Altering mRNA stability has been implicated in such cases as differentiation and development, hormone responsive systems, viral infections and the control of growth and the cell cycle. Several abnormal phenotypes have been attributed to changes in the steady state of a particular message, apparently as the results of mutations which effect changes in the half-lives of these messages. These aspects of the role mRNA stability can play in the life of a eukaryotic organism and in the cells of which it is made, will be critically reviewed to emphasize the significance of RNA stability in the control of gene expression.

In the literature "mRNA" is not well defined. According to Lewin, (1982), messenger RNA (mRNA) is cytoplasmic RNA that is associated with polysomes and extractable from polysomes with EDTA. The term mRNA has been used to refer to total cytoplasmic RNA, poly(A)+ cytoplasmic RNA, poly(A)+ whole cell RNA, or even total cellular RNA. The most acute point of confusion is the equation of poly(A)+ RNA with mRNA. Polyadenylation occurs in the nucleus, a cellular compartment devoid of translational

machinery. Translation of mRNA in the cytoplasm does not require polyadenylation and, indeed, many mRNA species lack poly(A) segments entirely. Those results obtained from experiments using total RNA cannot necessarily be directly compared with results using cytoplasmic RNA because nuclear RNA decays much more rapidly than does cytoplasmic RNA. In cases, where half-lives are hours long, it is unlikely that the nuclear component is influential. In most cases, in which the RNA is stable and any RNP component is likely to be small, this semantic confusion is merely that. However, in cases where half-lives are less than an hour, the nuclear component can have an impact. In addition, depending upon the state of the cell, cytoplasmic RNP components of certain RNA species are likely to vary. It is most important to consider these points in order to illustrate the lack of consistency in the literature. The experiments performed in this thesis consider cytoplasmic RNA only. In the following review of the literature, the discussion will consider the type of RNA that the authors actually analysed. Table Introduction A summarizes the term used by each set of authors to refer to the RNA prepared and the type of RNA that was actually prepared.

Steady-State:

Before the advent of recombinant DNA technology, the study of RNA decay, particularly of component species, was difficult, if not impossible. In the early 1970's, though cytoplasmic RNA was thought to display complex kinetics of decay, half-lives of RNA were studied mainly by analyzing populations of RNAs. In HeLa cells, one such population decays with a "short" half-life of 7 hours, whereas another longer-lived population decays with a half-life of 24 hours (Singer and Penman, 1973). In vegetatively growing cells of Dictyostelium discoideum, one such population

Table Introduction A

Reference	Stated type of RNA isolated	Actual type of RNA isolated
Abelson et al. (1974)	cytoplasmic mRNA	cytoplasmic RNA
Aviv et al. (1976)	globin mRNA	globin cytoplasmic poly(A)+ RNA
Alterman et al. (1984)	mRNA	cytoplasmic RNA
Bastos et al. (1977)	mRNA	cytoplasmic RNA
Brock and Shapiro (1983)	cytoplasmic mRNA	cytoplasmic RNA
Carneiro and Schibler (1984)	cytoplasmic mRNA	cytoplasmic poly(A)+
Casey et al. (1983)	mRNA	total poly(A)+
Chung et al. (1981)	cytoplasmic mRNA	cytoplasmic poly(A)+
Eick et al. (1985)	RNA	cytoplasmic RNA
Gelfand and Smith (1983)	nuclear RNA	nuclear RNA
Graves et al. (1987)	mRNA	total RNA
Guyette et al. (1979)	mRNA	total poly(A)+
Heintz et al. (1983)	mRNA	total RNA
Hereford et al. (1981)	mRNA	total RNA
Kelly et al. (1985)	mRNA	total RNA
Kelly et al. (1987)	mRNA	total RNA
Kidder and Pedersen (1982)	mRNA	cytoplasmic poly(A)+
Kimura et al. (1986)	mRNA	total RNA and total poly(A)+
Krowczynska et al. (1985)	mRNA	cytoplasmic RNA
Lowenhaupt and Lingrel (1978)	globin mRNA	globin cytoplasmic poly(A)+
Mangiarotti et al. (1985)	mRNA	cytoplasmic poly(A)+
Maquat et al. (1981)	globin mRNA	total RNA
Maquat and Kinniburgh (1985)	globin RNA	total globin RNA
Morris et al. (1986)	mRNA	total RNA
Perry and Kelley (1973)	mRNA	mRNA
Rabbitts et al. (1985)	mRNA	cytoplasmic RNA
Ross et al. (1974)	globin mRNA	poly(A)+ mRNA
Ross and Pizarro (1983)	mRNA	total RNA
Santiago et al. (1986)	mRNA	total RNA
Singer and Penman (1973)	mRNA	cytoplasmic RNA
Sittman et al. (1983)	mRNA	total or cytoplasmic RNA
Volloch and Housman (1981a,b,c, 1982)	cytoplasmic globin mRNA	cytoplasmic globin RNA
Wilson and Darnell (1981)	cytoplasmic mRNA	cytoplasmic poly(A)+
Wiskocil et al. (1980)	mRNA	total poly(A)+
Wolffe et al. (1985)	mRNA	total RNA and total poly(A)+

of poly(A)+ total RNA decays with a half-life of 50 minutes, the other with a half-life of 10 hours (Casey et al., 1983).

Recombinant DNA technology has enabled dramatic advances to be made in the understanding of component species of these populations. Carneiro and Schibler (1984) isolated 10 cDNA clones (pLC1-10) from mouse L-cells that hybridize to variously abundant poly(A)+ RNA species of several tissues. These 10 putative house-keeping genes are transcribed at disparate rates that do not correlate with their steady state levels of RNA, as determined by in vitro nuclear run off experiments and dot blot analyses, respectively. Rough kinetic labeling experiments were performed in which total poly(A)+ RNA, after 3 and 24 hours of labeling with [^{32}P]orthophosphate, was isolated and hybridized to the 10 clones. The results of these experiments suggest that the RNA species are degraded differentially, since the more abundant RNAs accumulate to a greater extent than do the rarer RNAs between the two labeling times.

The rate at which an RNA is degraded is only one factor in a complex interaction between synthesis, processing, and degradation of RNA and protein, the end product of which results in a given level of protein. One may assume that the level at which such control has been imparted is significant. As a result different RNA species are degraded at a wide variety of rates. Cellular myc and cellular fos oncogene mRNAs are said to persist in the cell with a half-life of 10-15 minutes (Dani et al., 1984; Greenberg and Ziff, 1984; Rahmsdorf et al., 1987). If a protein is required transiently and cannot be tolerated by the cell for long periods of time, it is necessary that both the protein and the message be short-lived. Vitellogenin RNA remains stable in the cytoplasm of Xenopus liver cells in the presence of estrogen with a half-life of 3 weeks (Brock and Shapiro, 1983). How do these mRNAs decay at such different rates? Is

there a particular region, local sequence or local secondary structure which signals to the cellular machinery to degrade the RNA at a given rate, or does the characteristic half-life of a message result from some signal conveyed by the overall structure of the mRNA in its native or RNP form?

Perturbations:

For many cellular functions and responses a change in the stability of a particular mRNA has been implicated in effecting, or helping to effect, a rapid, complete or appropriate change in the amount of encoded protein. It is assumed, in most cases, that the structure of the message itself is unchanged and that some component of the cellular milieu is responsible for causing the resultant change in half-life.

The expression of P_1 450 and P_3 450 genes in mouse liver and extrahepatic tissues rises in response to various mutagens, carcinogens, teratogens, and toxins, as the products of these genes are involved in the detoxification of various harmful agents. In response to dioxin the transcription of hepatic P_1 450 mRNA rises 8-fold, though the steady state of the total poly(A)+ RNA increases 27-fold, and the transcription of renal P_3 450 rises 2-fold though, the steady state level of mRNA increases 12-fold (Kimura et al., 1986). In these experiments the effect of RNA stabilization is inferred, as an increase in transcription cannot account for the increase levels of total poly (A)+ RNA. It is unclear as to whether the stabilization occurs in the nucleus, the cytoplasm, or both.

Early in adenoviral infection of HeLa cells, two viral transcripts from region 1A and two viral transcripts from region 1B all have short cytoplasmic half-lives of 6-10 minutes. Late during infection there is roughly a 10-fold increase in half-life of both 1A transcripts and of one of the two 1B transcripts. The longer transcript from region 1B remains

short-lived, which serves as an internal control showing that all cytoplasmic poly(A)+ transcripts are not becoming more long-lived (Wilson and Darnell, 1981). These authors actually quantitate the half-life of the cytoplasmic poly(A)+ RNA using approach to equilibrium. (See Chapter ID on Experimental Approaches.)

Hormones:

Of the many examples of hormonal effects on RNA stability a few will be discussed. Many hormone-responsive systems act by effecting an increase in the concentration of specific proteins. Guyette et al. (1979) showed that casein RNA in mammary explants was transcribed 2-4 fold more rapidly and was 17-23 times more stable in the presence of prolactin. The authors claimed that the hormone-induced stabilization was selective for total casein poly(A)+ RNA as pulse-chase protocols showed only a 1-4 fold increase in the half-life of bulk poly(A)+ RNA. The short-lived component was increased in half-life from 3.3 to 12.8 hours, while the long-lived population changes from 29.3 hours to 39.2 hours. For bulk RNA the decay curve reflects the contribution of many component species of RNA, so by looking at overall change of cellular RNA half-life, these authors are neglecting the possibly more dramatic changes of other component species, as in the case of casein RNA.

A case of RNA stabilization in response to hormone has been studied using the liver of Xenopus laevis. In the absence of estrogen, vitellogenin is not transcribed. Upon secondary estrogen (estradiol 17β) stimulation, vitellogenin is transcribed rapidly. Pulse-chase experiments exhibit a half-life of vitellogenin cytoplasmic RNA of about 3 weeks in the presence of estrogen. When hormone is removed, the RNA's half-life is reduced to that of bulk poly(A)+ mRNA in the presence or absence of

estrogen (16 hours), but is restabilized upon readdition of estrogen (Brock and Shapiro, 1983). In avian liver, vitellogenin and apoVLDLII poly(A)+ RNAs behave quantitatively similarly to each other and resemble qualitatively the behavior of Xenopus vitellogenin. Neither apoVLDLII nor vitellogenin RNA is detectable in avian liver prior to estrogen stimulation. Upon primary stimulation both transcripts rise and then seem to decay with half-lives of 22-26 hours (Wiskocil et al., 1980). The fact that these avian RNAs decay at any detectable rate in the presence of estrogen, given the 3 week half-life of Xenopus vitellogenin in the presence of estrogen, could be due to the fact that Brock and Shapiro (1983) used a Xenopus system, whereas Wiskocil et al. (1980) used an avian system, or that Brock and Shapiro (1983) used primary culture while Wiskocil et al. (1980) used in vivo culture, or that Brock and Shapiro (1983) used cytoplasmic RNA and Wiskocil et al. (1980) used total RNA, or that Brock and Shapiro (1983) employed secondary stimulation, while Wiskocil et al. (1980) used primary estrogen exposure or, most likely, that Brock and Shapiro (1983) continually changed the media containing the estrogen stimulus, whereas Wiskocil et al. (1980) used a single in vivo bolus of estrogen which may decay after a short time.

Some hormone-responsive systems act by effecting a decrease in the concentration of specific proteins, or even by effecting a decrease in the concentration of some and an increase in the concentration of others. Wiskocil et al. (1980) demonstrated that serum albumin is not affected by estrogen stimulation or withdrawal, yet the data of Wolffe et al. (1985) contradict this. The latter authors claim that in Xenopus liver, albumin RNA is turned off at the transcriptional level as well as destabilized 3-fold following estrogen treatment. After roughly 30 hours in the presence of estrogen the steady state level of albumin begins to return to

prestimulated levels, though the transcription level returns after about 6 hours. These contradicting data could be explained by the fact that Wiskocil et al. (1980) studied avian tissue and Wolffe et al. (1985) studied Xenopus tissue, or that Wiskocil et al. (1980) used and in vivo bolus, where as Wolffe et al. (1985) and Brock and Shapiro (1983) refreshed the media on their tissue culture at regular intervals.

Development and Differentiation:

Bulk changes in overall RNA metabolism have been detected as early in ontogeny as oocyte maturation. Total Xenopus oocyte RNA becomes stabilized following germinal vesicle breakdown in the presence of progesterone as the eggs progress from arrest in the first meiotic prophase (Gelfand and Smith, 1983). Kidder and Pedersen (1982) performed well controlled pulse-chase experiments in early mouse embryoes that showed that cytoplasmic poly(A)+ RNA of morulae (65-68 hours post-fertilization) decays with a half-life of 9.5 +/- .9 hours, but cytoplasmic poly(A)+ RNA of blastocysts (92-94 hours post-fertilization) decays with a half-life of 19.2 +/- 6.9 hours.

Changes in RNA stability have been shown to be involved in the life-cycle of the slime mold Dictyostelium discoideum. Upon starvation, and accompanied by pulses of cAMP, amoeboid cells aggregate to form mounds of cells, which eventually develop into a fruiting body, consisting of spore and stalk cells. The spore cells germinate, under appropriate conditions, into vegetatively growing cells which can then restart the cycle. As the cells aggregate, forming requisite cell-to-cell contacts, there is a 10-fold stabilization of developmental, aggregation-stage cytoplasmic poly(A)+ RNAs that are synthesized throughout the life cycle, but which do not accumulate until this stage (Mangiarotti et al., 1985). These authors did not show any non-developmentally regulated RNA whose half-life remained

unchanged. This would demonstrate that the transcript stabilization was specific to aggregation-stage transcripts and not representative of an overall effect on metabolism. Upon artificial disaggregation, these RNAs are again destabilized, unless cAMP is added to the medium. In this experimental system, constitutive cytoplasmic poly(A)+ species are not concomitantly destabilized (Chung et al., 1981; Mangiarotti et al., 1983), suggesting that the normal biological stabilization is, in fact, specific to aggregation-controlled messages.

In several differentiation model systems, there is notable evidence that a change in RNA stability permits a more efficient, if not the sole, mechanism for attaining a terminally differentiated phenotype, which often entails the massive accumulation of one or more mRNAs/proteins. Murine Erythroleukemia (MEL) cells is an in vitro mouse system for erythropoiesis, in which cells can be induced to differentiate into red blood cells containing huge quantities of globin mRNA/protein. Lowenhaupt and Lingrel (1978) noted a change in stability of globin cytoplasmic poly(A)+ RNA from a half-life of greater than 50 hours to one of 17 hours upon stimulation to differentiate. This was unexpected in light of the increased accumulation of globin as red cells mature. Volloch and Housman (1981a,1982) showed that physical stabilization is required to prevent end-stage lysis of these fragile MEL cells. With these culture conditions MEL cells can be observed to have morphological characteristics of mouse orthochromatophilic erythroblasts and reticulocytes (Volloch and Housman, 1981a, 1982). These authors used their system to establish that the half-life of globin cytoplasmic RNA is greater than 60 hours in terminally differentiated MEL cells (Volloch and Housman, 1981b). Aviv et al. (1976) postulated that the most effective manner in which to achieve the requisite accumulation of globin RNA so that globin protein synthesis is 90% of total, would be by

the bulk destabilization of non-globin transcripts. Using pulse-chase experiments with ^{3}H-uridine, Volloch and Housman (1981c) showed that bulk non-globin cytoplasmic RNA decays with a half-life of about 27 hours in uninduced MEL cells, 22 hours in 48 hour induced MEL cells, 13 hours in 96 hour induced cells and 5 hours in 120 hour induced cells, whereas globin remains stable. Krowczynka et al. (1985) showed that specific species of cytoplasmic RNAs are destabilized by 48 hours of induction. The half-life of actin cytoplasmic RNA changed from 16-26 hours prior to induction to 4-9 hours with inducer. The half-life of tubulin cytoplasmic RNA changed from 7 hours prior to induction to 3-4 hours with inducer. The degradation of two other RNAs was detected after induction whereas these messages were stable prior to treatment with inducer. Only globin was stable in induced cells (Krowczynska et al., 1985). Strangely, bulk poly(A)+ RNA was shown not to be destabilized during induction, and the effect of inducer on bulk poly(A)- RNA was not described. It is implied that it is either the poly(A)- message that is degraded upon induction or that the lack of bulk destabilization viewed by Krowczynka et al. (1985) was due to its offset by the massive increase of stable globin RNA.

Growth Control During the Cell Cycle:

In yeast cells (Hereford et al., 1981), human HeLa cells (Heintz et al., 1983), mouse lymphoma, S49, cells (Sittman et al., 1983), and mouse MEL cells (Alterman et al., 1984), DNA synthesis was shown to be coupled to histone RNA degradation in the presence of DNA synthesis inhibitors. Literature which describes sequences which control this programmed degradation suggest that such sequences exist both in the 5' untranslated region and the 3' untranslated regions of histone transcripts. The first 20 bases of the human histone H3 transcript, which is degraded

preferentially in the presence of DNA synthesis inhibitors, was replaced by the first 89 bases of the Drosophila hsp70 RNA (Morris et el., 1986). Total cellular RNA of this chimeric molecule did not degrade with characteristic histone kinetics. No control was performed, however, to demonstrate the appropriate regulation of histone RNA under heat shock conditions used to induce the chimeric, hsp70-histone H3, RNA. When the 20 bases of the H3 leader replaced the human β-globin leader, that fusion gene did exhibit histone-like degradation kinetics. In addition, the 3' hairpin loop must be present in the 3' untranslated region of the histone genes in order to undergo histone-like degradation in the presence of DNA synthesis inhibitors (Graves et al., 1987).

Mutants:

In several cases, mutant alterations in RNA structure have been associated with changes in the half-lives of specific species of RNAs. In the following cases, cellular transcripts from natural human mutants, which give rise to human disease, were analyzed to understand the nature of the disease.

Maquat et al. (1981) examined β^0 thalassemias in four Kurdish Jews whose homozygous disease made them globin-deficient. It was shown that total globin RNA from non-thalassemics was stable during a 30 minute actinomycin D chase, but that 30%-75% of the total globin RNA from the thalassemics had disappeared during this time period. (See Chapter ID Experimental Approaches.) These authors actually studied total RNA, since they lysed nucleated cells, though they claim to have studied globin mRNA. In this case, since the half-life of the mutant globin is roughly 30 minutes, the impact of the nuclear component could be significant. However, since their chase is so short (30 minutes) both mutant and normal

globin half-lives could be shorter than expected without changing the result that the mutation has decreased the half-life of the RNA. Kinniburgh et al. (1982) later sequenced the β-globin gene of the same patient and showed that a single nucleotide deletion at codon 44 produced a UGA termination codon at amino acid 60. These authors stress that the instability of the RNA may be unrelated to its ability to be translated (see Chapter IB General Features that Affect Stability), but may, in fact, result from a change in structure that would alter the susceptibility to nucleases. Another β^0 thalassemia (β^0-39) has a premature termination codon at amino acid 39, but shows no alteration in RNA stability (Humphries et al., 1984; Takeshita et al., 1984). This RNA is defective in transcription and/or early post-transcriptional processing. In contrast to the results of Kinniburgh et al. (1982), Humphries et al. (1984) and Takeshita et al. (1984) show that upon cotransfection with the appropriate suppressor tRNA, which would restore its translational potential, normal globin RNA levels are restored.

Burkitt's lymphomas and mouse plasmacytomas are often caused by altered c-myc expression. It is thought that the malignant phenotype is a result of the presence of more and/or aberrant, growth controlling c-myc transcript due to its increased stability. Two cell lines derived from Burkitt's lymphomas carry the t(8;14) translocation in which the immunoglobulin μ-heavy chain switch regions have been fused in a head to head orientation with the c-myc gene. Several aberrant transcripts are formed by starting on the anti-sense strand of the immunoglobulin region. All of these cytoplasmic RNAs have half-lives of several hours, in contrast to normal myc transcripts, which decay with a half-life of roughly 15 minutes (Eick et al.,1985). In these cases, it is possible that the increase in half-life is due to the addition of μ-heavy chain anti-sense sequences.

In a colon carcinoma cell line (COLO 320), one chromosome carries a normal c-myc gene, while the other one carries a c-myc gene in which the first exon has been deleted. Rabbitts et al. (1985) stated that cytoplasmic RNA transcribed from the normal gene decays at an expected half-life of 30 minutes, while the gene lacking the first exon decays substantially more slowly. It has been suggested that this first exon harbors target sequences for the rapid and specific degradation of the RNA, yet a fusion transcript with this first exon of myc contiguous to CAT (chloramphenicol acetyl transferase) sequences was unable to destabilize the CAT transcript (Piechaczyk et al., 1986). Both the hybrid transcript and the CAT transcript itself are stable over a period of 4 hours. These results suggest that the destabilizing ability of the first exon must be considered in the context of the entire message. Another prospect for the role of the first exon was described by Saito et al. (1983). These authors suggested that the transforming capacity of genes that have lost the first exon is due to the loss of the putative, stable stem-loop structure which probably encompasses the initiator AUG, which under normal circumstances, translationally controls expression.

A Technical Aside:

The above sampling of the literature on the topic of RNA stability clearly demonstrates complexities worthy of further study toward the understanding of the basic workings of the cell. In addition to questions of basic biology, more practical applications can be assigned to this research. In attempting to express a protein for large-scale production, short-lived messages can present a problem. A sequence that is known to stabilize RNAs could be added on to the encoding message to prolong its life in the cytoplasm, enabling the production of more protein per RNA. A

bacterial counterpart to this exists in two expression vectors that contain the promoter region and start codon of T4 gene 32 (Duvoisin et al., 1986). In this case, the prolonged stability of the hybrid message enables the selective labeling of the encoded polypeptide. Such a system would be attractive for mammalian expression vectors.

General Features that Affect Stability

Most studies of mRNA stability indicate that mRNA decays stochastically, behaving with first-order kinetics (Perry and Kelley, 1973; Lewin, 1980). Such kinetics imply that the degradative rate of the RNA is not related to the age of the RNA. That is, each RNA of a given type has an equal chance of getting destroyed at any given moment. There are many features of the RNA and cellular milieu, however, that can potentially affect the rate at which it is degraded. Growth rate and the state of confluence of the cell are suggested to affect RNA degradation rates. Characteristics of an RNA which may relate to its half-life include methylated cap, poly(A) tail, length, and translatability.

Growth Rate and State of Confluence:

Work on the effect on RNA degradative rates by the state of confluence and growth rate of the cells has yielded contradictory results. Perry and Kelley (1973) have supplied weak evidence that the doubling time of cells is directly related to the half-lives of RNAs in that cell. Mouse L-cells growing with a doubling time of 13.5 hours have an average half-life of decay of bulk RNA of 15 hours. The same cells growing with a doubling time of 41 hours have an average half-life of decay of bulk RNA of 42 hours. The experimental design used to effect the change in growth rate, however does not allow a fair comparison of the two growth rates. The faster growing cells were grown at 37^{o}C, and the slower growing cells were grown at 30^{o}C. Since enzyme catalysis rates are known to be directly related to temperature, it is easy to interpret these results by accounting for the increased rate of degradation with the more rapid catalytic rate of degradative enzymes at higher temperatures.

Since growth rates of cells usually slow as cells become more confluent it is conceivable that the state of confluence affects the degradative rate. Greenberg (1972) claims that non-growing cells have an intrinsically higher rate of mRNA degradation than do growing cells. Abelson et al. (1974) claim that confluent 3T3 and 3T6 cells degrade their RNA at similar, if not identical rates, while in exponential growth phase. They do state, however, that ribosomal RNA (rRNA) is unstable in confluent cells compared with its stable existence in growing cells. Possibly, this relates to the fact that (2'-5')oligoadenylate is higher in confluent cultures of Vero and L cells than in subconfluent cultures (Creasey et al., 1983). This oligonucleotide is an activator of latent RNase L and results from treatment with interferon. As will be discussed in Chapter IVC on the fos promoter, it is possible that the observed increased degradation of rRNA is due to the activated RNAse L in confluent cells. Dean and coworkers (Dean et al., 1986) have suggested that the increased level of c myc RNA that is seen in subconfluent Balb/c 3T3 cells compared to confluent cells is due to stabilization of the RNA. So that while the state of confluence may not have an effect on the stability of bulk RNA (Abelson et al., 1974), certain species may be affected (Dean et al., 1986).

Translation:

There is much evidence in the literature that implicates translation and the "protection" of ribosomes, as having at least a partial role in the determination of the stability of an RNA transcript. Losson and Lacroute (1979) showed in yeast that nonsense mutations seemed to destabilize the message, the farther upstream the mutation occurred. The presence of tRNA suppressor genes permitted the return to wild-type half-lives, implying that the interruption of translation affects the stability of the message,

and that this effect is more dramatic the more 5' the mutation occurs. Graves et al. (1987) have shown that translation is required for the experimentally defined degradation of histone RNA during inhibition of DNA synthesis, as a short insertion resulting in a termination codon blocks this controlled degradation. In Dictyostelium discoideum, Kelly et al. (1987) showed that developmentally regulated spore-germination specific transcripts are stabilized in the presence of cycloheximide, which prevents translational elongation, but not translational initiation. The transcripts, therefore, become covered with ribosomes and it could be argued that the stabilization resulted from protection of the RNAs from nuclease attack. In the presence of pactamycin, which blocks initiation of translation and results in broken-down polysomes, there was no effect on the stability of the RNA. Here, it could be argued that there is no increase in rate of degradation because the the putatively "bare" message would immediately be converted to an RNP.

There is also evidence which denies involvement of translation in the control of RNA stability. The β^0 thalassemic globin transcript (discussed above in Chapter IA6 Mutants (Humphries et al., 1984; Takeshita et al.,1984)), which has a nonsense codon at amino acid 39, was not destabilized by this mutation. It was, however, defective in nuclear stability and/or transport from the nucleus to the cytoplasm.

In other circumstances, stability can be acquired by the lack of protein synthesis. Superinduction is the enhancement of the amount of a given transcript in the presence of protein synthesis inhibitors. Messages appear to be stabilized because proteins, including a labile RNase, are not being produced. Histone cytoplasmic RNAs are stabilized in the presence of protein synthesis inhibitors prior to inhibition of DNA synthesis, whereas non-histone RNAs examined were not superinduced (Sive et al., 1984). Kelly

et al. (1983) showed that c-myc is superinduced in the presence of cycloheximide and mitogen, which supports the notion that a labile protein may regulate c-myc expression in these cells.

Polyadenylation:

There are conflicting, though potentially compatible, data on the role of the poly(A) tail in RNA stability. As messages enter the cytoplasm, the poly(A) tail consists of 300 A's, on the average. With time, the length of the tail is slowly decreased (Littauer and Soreq, 1982). In the case of globin, the existence of a poly(A) stretch of at least 32 bases affords the message protection from degradation when injected into Xenopus laevis oocytes; a tail containing fewer than 16 A's has no protective capacity (Nudel et al., 1976). A tail containing more than 32 bases is no better protected than one containing 32. Since an older message has an equal probability of being destroyed as a newer message, the length of a tail may enter into the decision of which RNA to degrade if the tail length falls below some critical number of residues. In addition to globin, Woodland and Wilt (1980) adenylated HeLa histone RNAs that were naturally lacking a poly(A) tail. Upon injection into Xenopus laevis oocytes these transcripts had a longer half-life than did the poly(A)- species. Synthetic RNAs from chicken lysozyme, calf preprochymosin and Xenopus globin were transcribed in vitro using SP6 and were shown to be more stable with a poly(A) tail (Drummond et al., 1985). Zeevi et al. (1982) indicated that newly formed RNAs synthesized in the presence of 3' deoxyadenosine (3' dA), which prevents poly(A) tail addition, are properly spliced and enter the cytoplasm correctly. Under these circumstances, messages are shorter-lived.

However, several examples exist in the literature in which the removal of the poly(A) tail did not affect the half-life of the RNA. Interferon RNA was unaffected by the removal of the tail (Soreq et al., 1981). Goto et al. (1981) showed that the length of the poly(A) tail was dependent upon the length of the RNA, and in no way correlated with the change in stability associated with particular messages in differentiating fetal calf myoblasts. In a recent study, it was shown that the poly(A) tail decreased the half-life of a message. Two c-myc messages from HeLa cells were shown to differ only in the presence of a poly(A) tail long enough to bind oligo(dT); they had otherwise identical structures. Oddly, it was the "nonpoly(A)+" species that decayed more slowly (t(1/2)>1 hour vs. t(1/2)=12 minutes) (Swartwout et al., 1987).

5' Terminal Cap:

The 5' terminal methylated cap structure (m^7GpppG^m) is also considered to have a role in protecting the RNA from degradation. This cap, like a poly(A) tail, would perform its protective function in a non-selective, non-discriminatory manner, such that it could afford a protective capacity only in its presence, and its presence or lack thereof must be signalled by particular features of the message. The 5' terminal methylated cap structure is attached to the 5' nucleotide of hnRNA early during transcription (Salditt-Georgieff et al., 1980). Furuichi and co-workers (1977) determined that the cap (both methylated and unmethylated) protects the message from 5' exonucleolytic attack. These authors injected radiolabeled reovirus RNAs with normal caps, demethylated caps (GpppG), and unblocked 5' ends (ppG), into Xenopus laevis oocytes and showed that the last category of RNA was degraded more rapidly. More recently Camper et al. (1984) showed that undermethylated cap structures did not affect the rate of degradation, a result consistent with Furuichi et al. (1977).

Length of the RNA:

Length of the RNA is purported to be a factor that controls its rate of degradation. On the average, long-lived transcripts tend to be short in length, while short-lived transcripts tend to be longer in HeLa cells (Singer and Penman, 1973), human lymphocytes (Berger and Cooper, 1975), and mouse L-cells (Meyuhas and Perry, 1979). In yeast, half-lives of 15 RNAs were determined to fall into two classes based on length of the cDNA (Santiago et al., 1986). (These half-life determinations were made using total cellular RNA. Since the decay rates ranged from 2 minutes to over 100 minutes, the contribution to RNA of nuclear RNA degradation is likely to have influenced these half-lives.) Apparently, the target size for attack by an RNase is greater the longer the transcript.

Mechanisms of Degradation

The rate at which an RNA is degraded is likely to be inherent in its primary structure (and/or resultant secondary structure), which itself can serve as a signal to cellular degradative components. This signal to the cell indicating the rate at which an RNA should be degraded could be mediated either through a form of protection of the message as would be assumed in the case of a long-lived RNA, or through a form of exposure of the message as would be indicated in the case of a short-lived message. Given the continuum of rates at which RNAs disappear, it is conceivable that a combination of protection and exposure are involved. This signal could directly cause the interaction of the RNA with a degradative RNase or could transfer the signal through one or more intermediate proteins or macromolecular complexes. Are these signals sequences or local secondary structures that occur at intervals over the length of the transcript? Are these signals the result of complex secondary or tertiary structural interactions between distant ends of the molecule? Or are both models involved in a complex trigger to degrade an RNA?

There is precedent to suggest that local secondary structure has functional significance. RNase studies have shown that the differential translatability of mouse and rabbit α- and β-globin RNAs could be due to differential secondary structure surrounding the start codon. Pavlakis et al. (1980) demonstrated that the AUGs of the α-globins, which are translated significantly less efficiently than are the β-globins, are nuclease-resistant and are therefore likely to be buried in tertiary structure or base paired in secondary structure. When the thymidine kinase gene was altered in its 5' untranslated region by insertion of an increasing number of oligonucleotides which potentially formed secondary

structure, the translational efficiency as assayed in rabbit reticulocyte lysates and wheat-germ extracts decreased as the number of oligomers increased (Pelletier and Sonenberg, 1985). Kozak (1986) clearly demonstrated that the insertion of an oligonucleotide that forms a stable hairpin loop (ΔG=-50 kcal/mol) in the 5' untranslated region of preproinsulin mRNA inhibits translation 85-95% when transfected into COS cells, yet a less stable hairpin loop (ΔG=-30 kcal/mol) has no effect. These data indicate that it is the secondary structure that is important, not the increased length supplied by the oligonucleotide.

There is also evidence for secondary structural interactions between distant ends of an RNA molecule. Using the combination of nuclease data and computer analysis Lockard et al. (1986) showed that long range interactions between the 5' and 3' untranslated regions of mouse β^{Maj} globin occur by the molecule folding back upon itself. Single-stranded regions were defined by S_1 and T_1 ribonuclease mapping, and double-stranded regions were defined by cobra venom V_1 ribonuclease mapping. The computer algorithm used predicted secondary structures of minimum free energy that were compatible with the nuclease data.

Since primary sequence is the basis for secondary and tertiary structure, a complete series of chimeric constructs between two RNAs could begin to address these issues. Does a particular small sequence or local secondary structure encode all that is necessary to signal the rate at which an RNA is degraded? Such a scenario was suggested by Kamen and Shaw (1986) in which a 5-mer, AUUUA, was assumed to be the important sequence of the 51-mer that destabilized rabbit globin. An AU-rich 51-mer from the 3' untranslated region of a human lymphokine RNA, GM-CSF, was inserted into the 3' untranslated region of rabbit β-globin; the chimeric RNA was degraded more rapidly than the normal β-globin RNA. Are additional

sequences necessary for a trigger such that many important sequences are located up and down the length of the RNA? Or are complex interactions involving both ends of the molecule of significance? This is implied by data of Piechaczyk et al. (1986) who showed that the non-coding exon of c-myc does not destabilize CAT, though its removal seems to stabilize the myc message in its own context (Rabbitts et al., 1985; Eick et al., 1985).

Another potential signal for degradation may involve an RNA intermediate. As will be discussed in Chapter IV, some growth factors in serum were shown to induce the production of β_2-interferons (Zullo et al., 1985; Kohase et al., 1986, 1987) which in turn induced the synthesis of (2'-5')oligoadenylate synthetase, leading to the production of (2'-5')oligoadenylate, a potent activator of latent RNase L. RNase L degrades single-stranded RNA most frequently after UA, UG and UU residues (Floyd-Smith et al., 1981) that are covalently attached to double-stranded RNA regions (Nilsen and Baglioni, 1979). Initially RNase L was thought to degrade only double-stranded viral RNAs, yet recently there is evidence that cellular RNAs (Jonak and Knight, 1984; Dani et al, 1985) and rRNA (Nilsen et al., 1982) can also act as substrates. Transcripts from B2 repeats accumulate in the cytoplasm when serum-starved mouse fibroblasts are growth-induced by serum (Edwards et al., 1985). Clemens (1987) pointed out that the 3' untranslated AU-rich regions of lymphokineshwhich contain the Shaw/Kamen 5-mer have striking complementarity to the AU-rich B2 transcripts. He suggested that these stretches of double-stranded RNA could protect RNAs from degradation. More likely is the possibility, given the characteristics and kinetics of RNase L and the sequence and kinetics of the B2 RNAs, that these short transcripts activate the degradation of genes having the Shaw/Kamen sequence. It would be most useful to assess the stabilities of these RNAs in the absence of serum refeeding in order to avoid the induction of the RNase L system.

Experimental Approaches for the Study of RNA Stability

RNA stabilities have been determined by a variety of methods. No method is flawless, each having its technical and/or interpretational difficulties. Several of the major methods will be described and critiqued. This discussion forms the rationale for the development of an improved system for the measurement of RNA stabilty. Each method involves a time course in which samples are taken at intervals and processed as explained.

^{3}H-uridine Pulse/Chase:

Many studies have been performed using transient labeling of RNA with ^{3}H-uridine followed by a chase with unlabeled uridine. To determine the half-life of bulk poly(A)+ RNA, isolated RNA is hybridized to oligo(dT)-cellulose, and the bound fraction is counted (e.g., Kidder and Pedersen, 1982). To determine the half-life of a given species of RNA, isolated RNA is hybridized to the corresponding cDNA on a filter, non-homologous RNA washed off, and the bound fraction counted (e.g., Krowczynska et al., 1985). As it is difficult to obtain sufficient label in a given species, this approach is limited to cases in which the RNA is metabolically active or one that accounts for a significant portion of the RNA of the cell.

Various parameters must be controlled in order to ensure an accurate determination of half-life. The labeling time of the pulse, when considering a particular species, must be significantly less than its half-life (Guyette et al., 1979). For each set of conditions, it must be shown that (1) label is incorporated in a linear fashion into that species (Guyette et al., 1979); (2) the specific activity of the pool size of the labeled precursor is constant during the extent of the chase (Bowman and

Emerson, 1980); (3) the chase is effective and rapid, disallowing continued incorporation into RNA; and (4) the labeled precursor is not reutilized once degraded (Schimke, 1970). To assist in averting the latter two problems, glucosamine is often added to the chase media to convert UTP to UDP-glucosamine (Lengyel, 1980).

Similar studies have been performed using density-labeled nucleosides instead of radiolabeled precursors (Bowman and Emerson, 1980). The transiently labeled RNAs are purified by density gradient equilibrium centrifugation and translated in vitro to determine the amount of the specific message present. In these experiments the kinetics of labeling and precursor pool-sizes must also be determined.

Kinetics of Approach to Equilibrium:

A method for studying RNA stability, which uses the kinetics of an approach to equilibrium is described by Greenberg (1972). Its use is limited to cells that are exponentially growing and to RNAs that remain at equilibrium level during the course of the experiment. The method is based on the concept that if an RNA is at steady state, the rate of synthesis must correspond exactly to the rate of decay, given that other processing rates are constant. The rate of degradation is determined by the rate at which incorporation reaches a maximum value. Therefore, the longer-lived is a message, the lower is the rate at which it reaches its maximum of incorporation.

Cells are continuously labeled with ^{3}H-uridine, during which time aliquots are removed and hybridized as detailed above for the ^{3}H-uridine pulse/chase approach. Not only is this approach limited in application, but there are several difficulties associated with it. The precursor pool must be monitored to ensure constant specific activity. In addition, a correction must be made for RNA processing time.

Use of Drugs to Arrest Transcription:

Various metabolic inhibitors have been employed to arrest transcription so that the RNA present at a given time can be followed as it decays. These include actinomycin D (Rabbitts et al., 1985; Piechaczyk et al., 1986), nogalamycin (Kelly et al., 1985), and daunomycin (Margolskee and Lodish, 1980). The RNA is then assayed in any of several ways. Translation products can be assayed in vivo, and the protein products quantified (Koch and Fressen, 1979). Interpretation would rest on the assumption that it is the message that is limiting and not any aspect of protein synthesis. In addition, such an approach would only determine the stability of mRNA and not any other component of total RNA. Translation products can be assayed in vitro using purified RNA. RNA present at a given time can be quantified using either Northern blot analysis or an S1-type analysis.

To determine the half-life of an RNA it is critical that no other parameter of cellular metabolism be affected that could impinge upon RNA stability. Protein synthesis ought not be altered to prevent any change in the synthesis of a labile RNase (as it is thought to be the basis of superinduction) or a labile protective molecule. Either of these could differentially affect specific RNAs. An inherent problem with this approach is that RNA synthesis itself should not be halted for the same reasons that protein synthesis should not be halted. How can RNA stability be determined under conditions that promote changes in the synthesis of molecules controlling it?

There is extensive literature, however, that details metabolic effects of these drugs, especially actinomycin D, in addition to the arrest of RNA synthesis. Singer and Penman (1972) showed that actinomycin D caused HeLa cell polysomes to sediment more slowly in sucrose gradients, implying that

mRNAs are more lightly loaded with ribosomes as the rate of translational initiation slows relative to the rate of translational elongation. Even low doses of actinomycin D have been shown to result in a decrease in protein synthesis (Schwartz, 1973). Steinberg et al. (1975) showed that tyrosine aminotransferase and its RNA were degraded more slowly in the presence of actinomycin D, and Cereghini et al. (1979) showed that actin RNA was degraded more slowly in the presence of actinomycin D. In HeLa cells, however, Singer and Penman (1973) showed that bulk cellular RNA decayed more rapidly in the presence of this drug. Furthermore, simply stressing the system to the point of decreasing cell viability by drug treatment would have a profound effect on stability (Schwartz, 1973).

Our Approach

An approach for the study of RNA stability has been designed and implemented which averts many of the problems of the classical methods described above. This method had no difficulties with quantity of RNA, pool sizes of the labeling molecule or kinetics of labeling. In addition, the manipulation of the input DNA permits specific sequences to be studied with respect to their effect on RNA stability. The strategy that was chosen was to quantify the decay of RNA after a brief, but intense pulse of transcription of a specific DNA driven by an inducible promoter. It was essential that a large amount of RNA be produced quickly in response to induction, that the promoter be turned off quickly after the pulse of transcription, and that there be a many-fold induction of RNA over basal, constitutive levels. The transiently expressed RNA was then followed quantitatively using Northern blot analysis. Chimeric DNAs between short-lived and long-lived messages can be expressed from the promoter in an attempt to isolate and define sequences important for signalling to the cell's degradative system the degradation rate of the message.

Human c-*fos* Promoter:

Several promoters were evaluated in this context. The promoter of the human c-*fos* gene proved most useful. Results pertaining to the human c-*fos* promoter will be described in detail in Chapter IV, to the *Drosophila* *hsp*70 promoter in Chapter II, and to the human *metallothionein* promoter in Chapter III. The human c-*fos* gene is induced to a high level of transcription rapidly in response to serum stimulation following a one to three day period of quiescence in platelet-poor plasma. This gene was also useful in that its transcript is short-lived and provided a convenient

short-lived counterpart for chimeras between messages of disparate half-lives. The transcript of the human β-globin gene was used as the long-lived message for the construction of the chimeras because (1) it is long-lived and (2) it is a major transcriptional product of MEL cells in which these studies were originally intended. Table Introduction B provides a chronological summary of the information to date about the half-lives of globin RNAs in various cell types. More details of the half-life of β-globin are discussed in the section on differentiation and development. It is striking, however, that the value of 17 hours is so recurrent. The half-life determined in these studies in Balb/c 3T3 cells is 17.5 hours.

Other Promoters Considered:

Two alternative promoters were considered prior to the initiation of experimental work, the interferon promoter and the mouse mammary tumor virus (MMTV) promoter.

The interferon promoter was rejected for two reasons. It had been demonstrated that endogenous interferon was produced and secreted from MEL cells during growth and differentiation (Friedman-Einat et al., 1982). Since these studies were initially intended to be performed in MEL cells, it was impossible to employ an inducer of transient expression that was actually produced by the cells. Interferon was also unacceptable for its known effect of indirectly inducing RNase L (Nilsen and Baglioni, 1982). As explained elsewhere, evidence is mounting that this RNase, which was originally considered to act only upon double-stranded RNA viruses, may also destabilize rRNA (Nilsen and Baglioni, 1982) and cellular RNAs (Jonak and Knight, 1984; Dani et al., 1985). Recently, several groups (Zullo et al., 1985; Kohase et al., 1986) have shown that growth factors, present in serum, induce the synthesis of β_2-interferon. Therefore, serum-mediated

Table Introduction B

Reference	ß-globin 1/2 life (hours)	cell type/ RNA	method of half-life determination
Gurdon et al. (1973)	as stable as host RNAs	frog oocytes	globin protein synthesis after microinjection of message
Ross et al. (1974)	> 10	MEL cells/ poly(A)+ mRNA	Actinomycin D chase
Aviv et al. (1976)	16-17	MEL cells/ poly(A)+ cytoplasmic RNA	^{3}H-uridine pulse/chase
Stacey and Allfrey (1976)	heterospecific mRNAs, mRNPs, polysomes, continued globin synthesis for >25 hours	Hela cells	immunoflourescence of globin proteins
Bastos et al. (1977)	17	mouse erythroid/ cytoplasmic RNA	^{3}H-uridine pulse/chase
Volloch and Housman (1981a,c)	> 60	BSA stabilized MEL cells/ cytoplasmic RNA	^{3}H-uridine pulse/chase
Ross and Pizarro (1983)	16.5	nucleated bone marrow cells/ total RNA	Actinomycin D chase

induction of fos may indirectly stimulate RNase L activation through simultaneous induction of β_2-interferon by growth factors.

The retroviral LTR promoter of mouse mammary tumor virus (MMTV) was also considered. This promoter was shown to be induced by glucocorticoids even when promoting heterologous DNA, yet the maximal induction over basal was insufficient for our purposes. Dihydrofolate reductase was driven by the MMTV promoter in response to glucocorticoids, but there was only a five-fold induction of message in response to inducer (Lee et al., 1981).

The promoters used in our initial studies seemed to have characteristics and kinetics more suited to requirements for the experimental design. The Drosophila hsp70 and the metallothionein II_A promoters had been shown to induce heterologous DNAs in heterologous cells. Details of the advantages of each promoter will be described in the Introductions to the next three chapters on the implementation of this approach.

Chapter II

Use of the Heat Shock Promoter

Introduction

Our first evaluation of an inducible promoter for the study of mRNA stability employed the Drosophila heat shock protein 70 (hsp70) promoter. Much evidence had accumulated which suggested that heat shock induction of transcription should work even across species lines given the conserved nature of the sequences from bacteria to man. For example, Corces et al. (1981) showed that Drosophila hsp70 gene was heat inducible at the level of transcription after transfection into mouse L cells. In these studies, a 3.6 kb segment of Drosophila DNA which carried 1.1 kb of 5' flanking DNA and .2 kb of 3' flanking DNA was able to respond to heat in the heterologous cell. Pelham and Bienz (1982a) showed that the Drosophila hsp70 gene was heat inducible in monkey COS cells and Voellmy and Rungger (1982) showed that Drosophila hsp70 gene was heat inducible in Xenopus oocytes. Thus the Drosophila hsp70 promoter, whose non-inducible temperature is 25°C and whose heat shock temperature is 37°C, could respond properly to conditions for mammalian cells, 37°C and 42-43°C, respectively.

The heat shock promoters of Drosophila have been intensively studied and precisely mapped. The upstream regions of four Drosophila heat shock genes, (hsp83, hsp70, hsp68, and hsp26) were sequenced and shown to have a common region of dyad symmetry centered roughly 25 bases 5' of the TATA box (Holmgren et al., 1981). Pelham (1982) showed that this inverted repeat appears in hsp22 and hsp27 as well. In addition, there is a consensus sequence common to this region of which 10 of the 14 bases form a smaller, off-centered inverted repeat. Pelham and Bienz (1982b) placed a synthetic version of this consensus sequence upstream of the herpes simplex virus thymidine kinase gene, and found that the hybrid gene was transcriptionally responsive to heat shock in monkey COS cells and Xenopus oocytes. Dudler and Travers (1984) extended this by showing that only a low level of heat

induction occurs when just the consensus sequence is present, but when sequences further upstream, to -97, were used, maximal heat induction was obtained. (In our studies, the CAT and myc constructs described below each have about 1kb of Drosophila hsp70 DNA upstream of the transcriptional start.)

The literature strongly suggested that the stability of bulk RNA is unaffected by heat. Kruger and Benecke (1982) showed that the half-life of bulk RNA in Drosophila cells grown at 25°C was not affected by a one hour heat shock at 37°C. In HeLa cells, after a one hour heat shock at 42°C, protein synthesis returns to control values rapidly without further RNA synthesis (McCormick and Penman, 1969). In vitro translation of control and heat shock cytoplasmic RNA of HeLa cells resulted in no large changes in the concentration of control polypeptides, implying that the 37°C RNAs are still present at the same levels (Hickey and Weber, 1982a,1982b). Clearly, the half-life of bulk RNA is not affected by heat treatment.

Results

CAT Activity in 3T3 Cells:

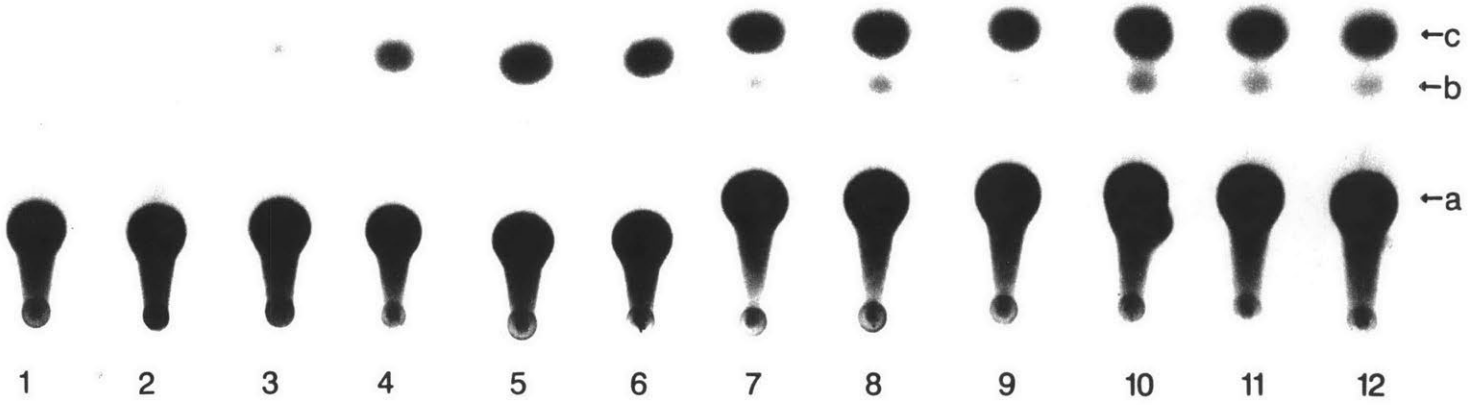
In order to assess the usefulness of such an approach, it was important to determine that a transcript driven by the heat shock promoter would exhibit inducible behavior. The system was first tested with an easily assayed product, CAT (chloramphenicol acetyl transferase), in cells which were easy to transfect (3T3). CAT activity was assayed by incubating cell lysates with ^{14}C-chloramphenicol and acetyl CoA and, after purifying extractions, and then running the incubation mixture on thin layer chromatography. If CAT activity were present, acetylated forms of chloramphenicol would be visible on autoradiographs of the chromatographs. (See Figure HSPB.) A construct containing the Drosophila hsp70 promoter driving the bacterial CAT gene (HSPCAT-1) was obtained from Igor Dawid's laboratory via Mary Lou Pardue's laboratory. (See Figure HSPA, modified from DiNocera and Dawid, 1983). (Figures of all constructs are located at the end of each chapter.) The expected transcript carried a 65 base transcriptional leader of Drosophila hsp70. Five μg of HSPCAT-1 were cotransfected (Method A) into NIH/3T3 cells along with 1 μg of PMSVNEO (Figure HSPA) (a molar ratio of 10:1) which contains the bacterial gene neo^R (TN5), a useful mammalian selectable marker. The drug G418 is phosphorylated and inactivated by the protein product of the neo^R gene; cells expressing this gene are thus resistant to the drug. Five colonies from each of the two plates which had been transfected were cloned out using cloning cylinders. All ten cell lines were screened for CAT activity following a 2 hour heat shock at 42.5^{o}C. .01 units of CAT enzyme were used as a positive control and untransfected NIH/3T3 cells were used as a negative control in all CAT assays. Six of the 10 clones showed a significant portion of chloramphenicol substrate which had been shifted to acetylated forms.

The first experiments were designed to determine if CAT gene activity could be induced from non-detectable levels to high levels and then allowed to decay. A time course experiment of heat shock at 43°C with increasing lengths of chase at 37°C was performed on one of the six CAT-positive clones. Twelve 100mm tissue culture plates, seeded with cell line 1B1A3, were grown to confluence. Each plate of cells received a two hour heat shock, or one hour in the case of lane 2, of Figure HSPB, and chased at 37°C. At t=0 cell lysates were made and stored at -20°C for assaying. Figure HSPB shows the level of CAT activity for each time point, and demonstrates that the protein activity responded to heat induction. There was no detectable activity at t=0, it rose steadily until about t=5 to t=7 and then began to drop off. No quantitation was performed. This experiment was not normalized in any way, (e.g. to protein), so any variation from the steady rise of activity or from the steady fall of activity could easily be due to experimental error. Total RNA showed similar induction (data not shown).

myc RNA in 3T3 Cells:

The next series of experiments involved the study of myc RNA being driven by the heat shock promoter, since this RNA has several features which make it amenable to stability studies in this system. The first exon, which has no protein coding capacity, appears to be responsible for signalling to the cell's degradative enzymes to destroy the message (Rabbitts et al., 1985; Eick et al., 1985). (Refer to Chapter IA6 Mutants.) In addition, since myc is a short-lived message it would be useful for making chimeric constructs with a long-lived message (e.g. globin). These chimeric constructs could then be used for assessing sequences encoding stabilizing or destabilizing capacities. Two myc

Figure HSPB: Time course of CAT activity in a 3T3 cell line cotransfected with PSV2NEO and HSPCAT-1. Twelve confluent 100mm plates with cell line 1B1A3 were either not heat shocked (lane 1), heat shocked for 1 hour at 43°C with no chase (lane 2), or heat shocked for 2 hours at 43°C with 0 (lane 3), 1 (lane 4), 2 (lane 5), 3 (lane 6), 4 (lane 7), 5 (lane 8), 6 (lane 9), 7 (lane 10), 8 (lane 11) or 9 (lane 12) hours of chase at 37°C. Equal aliquots (35 μl) were incubated with ^{14}C-chloramphenicol and acetyl CoA at 37°C for 45 minutes. Ethyl acetate extracted samples were run on TLC. The figure represents an autoradiograph of the chromatograph in which the parent substrate and the modified forms are as marked by the arrows (a is chloramphenicol, b is 1-acetate chloramphenicol and c is 3-acetate chloramphenicol).


←c
←b
←a
1
2
3
4
5
6
7
8
9
10
11
12

plasmids were constructed which used the Drosophila heat shock promoter (Livak et al., 1978) to drive its transcription (Figures HSPC-HSPE). One construct contained all three exons, NEOHSP123MYC (Figure HSPD). The other contained only the two protein-coding exons, NEOHSP23MYC (Figure HSPE). Both plasmids carried the neo^R gene. Each transcript carried a 65 base heat shock promoter transcriptional leader.

Ten μg of plasmid DNA was transfected (Method A) onto each of 4 100mm tissue culture dishes containing NIH/3T3 cells, for each construct. After selection in G418, 24 cell lines putatively carrying each plasmid were cloned. Total RNA was isolated from 17 of the cell lines transfected with NEOHSP123MYC and from 12 of the cell lines transfected with NEOHSP23MYC. These cell lines were screened by an SP6 RNase protection assay. Fifteen and 11 cell lines tested positively from the NEOHSP123MYC and NEOHSP23MYC transfections, respectively. One experiment was done on one positive cell line carrying each myc construct, C3A1 for NEOHSP123MYC and D4B3 for NEOHSP23MYC. Six 150mm tissue culture dishes of each cell line were grown to confluence. One of each set was not heat shocked, the others were heat shocked at 43°C for 1 hour followed by 3, 4, 5, 6, or 8 hours of chase at 37°C. Figure HSPF shows Northern blots of these experiments in which 7 μg of cytoplasmic RNA was hybridized with the 1.3 kb Cla1-EcoR1 fragment of human c-myc (A and B). These blots were reprobed with mouse lactate dehydrogenase DNA (kindly supplied by J. Mendel) as a normalizing control (C and D). Quantitation and statistical analyses were performed as described in the Appendix. Table HSP shows the data from these experiments. Each experiment was run on two separate gels. All 4 gels, two from each experiment, gave statistically acceptable results, yet the half-lives extracted from the two sets of data were different and incompatible with expected values based on the literature. Values of 2

Figure HSPF: Heat shock induced transcription of NEOHSP123MYC (C3a1) and NEOHSP23MYC (D4b3). Cell lines C3a1 (A and C) and D4b3 (B and D) were either not treated with heat (lanes 1 and 7) or treated with heat for 1 hour at 43°C and chased for 3 (lanes 2 and 8), 4 (lanes 3 and 9), 5 (lanes 4 and 10), 6 (lanes 5 and 11) and 8 (lanes 6 and 12) hours at 37°C. Cytoplasmic RNA was run on a formaldehyde gel, transferred to Genescreen Plus and probed with HUCMYC DNA (A and B). The same Northern blots were reprobed with LDH cDNA for normalizing (C and D).

A
B
1 2 3 4 5 6
7 8 9 10 11 12
C
D
1 2 3 4 5 6
7 8 9 10 11 12

Table HSP

HSPMYCS

CONSTRUCT	CELL LINE	EXPT.	BLOT	1/2-LIFE±S.D.[5]	t[1]	D.F.[2]	P[3]	AVERAGE±S.D.[4,5]
123MYC	C3a1	4/9/85	4/22/85A	1.0±.2	4.7	3	.01<P<.025	
123MYC	C3a1	4/9/85	5/10/85A	2.9±.3	24.9	3	<.001	2.0±1.3
23MYC	D4b3	4/9/85	4/22/86B	3.9±.6	6.41	2	.01<P<.025	
23MYC	D4b3	4/9/85	5/10/85B	2.0±.0	150.0	2	<.001	3.0±1.4

1 t-statistic (See Appendix)
2 degrees of freedom (See Appendix)
3 P-value ≤ .05 is acceptable (SeeAppendix)
4 average of statistically acceptable half-lives
5 half-lives in hours

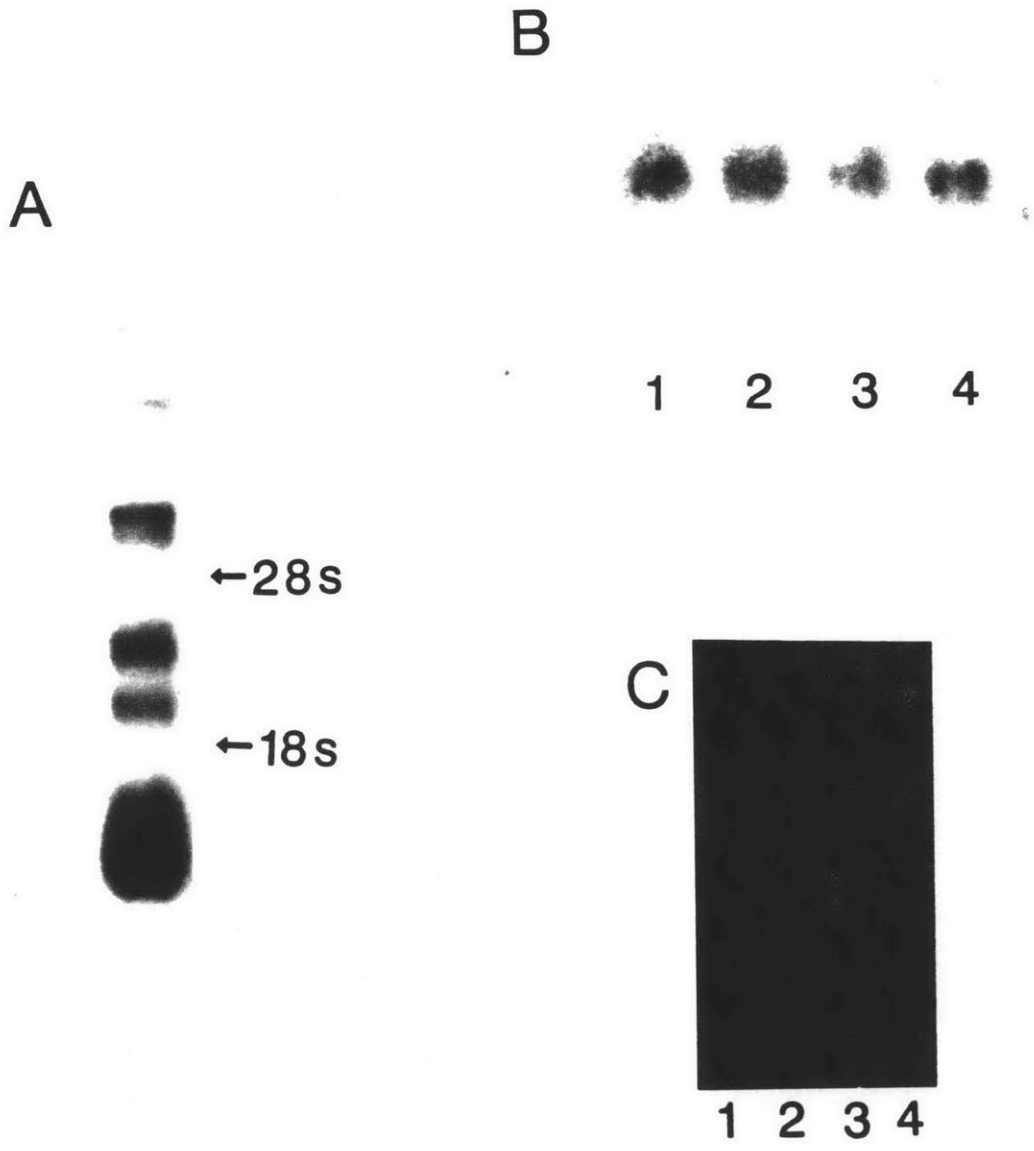
hours and 3 hours were obtained for HSP123MYC and HSP23MYC, respectively. Previously published reports on the half-life of myc RNA was on the order of 15 minutes (Dani et al., 1984).

CAT activity and CAT RNA in MEL cells:

Half-lives in two different cell lines, MEL and 3T3, were to be compared in order to address the question of whether RNA degradation was characteristic of the RNA in a given cell type or intrinsic to the RNA. Since MEL cells do not easily take up DNA by $CaPO_4$ coprecipitation, MEL cells which contained exogenous DNA were obtained by fusing cells with protoplasts made from bacteria harboring DNA of interest. Since protoplast fusion had not been successful with two different bacteria, it was necessary to place the selectable marker on the same plasmid as HSPCAT, creating NEOHSPCAT (Figure HSPA). Protoplast fusion was performed as described in Material and Methods. 24 G418-positive MEL cell lines were cloned out. 12 of the 18 cell lines screened, tested positively for heat-induced CAT activity.

Total RNA was isolated from HSPCAT 3a3 after 2 hours of heat shock at 43°C. The RNA was run on a Northern gel containing formaldehyde, transferred to GeneScreen Plus (New England Nuclear), and first probed with HSPCAT. As can be seen in Figure HSPG-A, this probe hybridizes with many high molecular weight species of RNA. In attempting to reduce this complex pattern to a single band for quantitation of RNA stability, other probes were tried. Since spurious hybridization might be due to unexpected transcriptional starts from the 3' LTR of NEOHSPCAT or cross-hybridization with endogenous heat shock transcriptional leaders, the blot was reprobed with purified insert containing only CAT sequences, (1.62 kb, Hind3 to BamH1). No change in hybridization was observed. The total RNA was poly

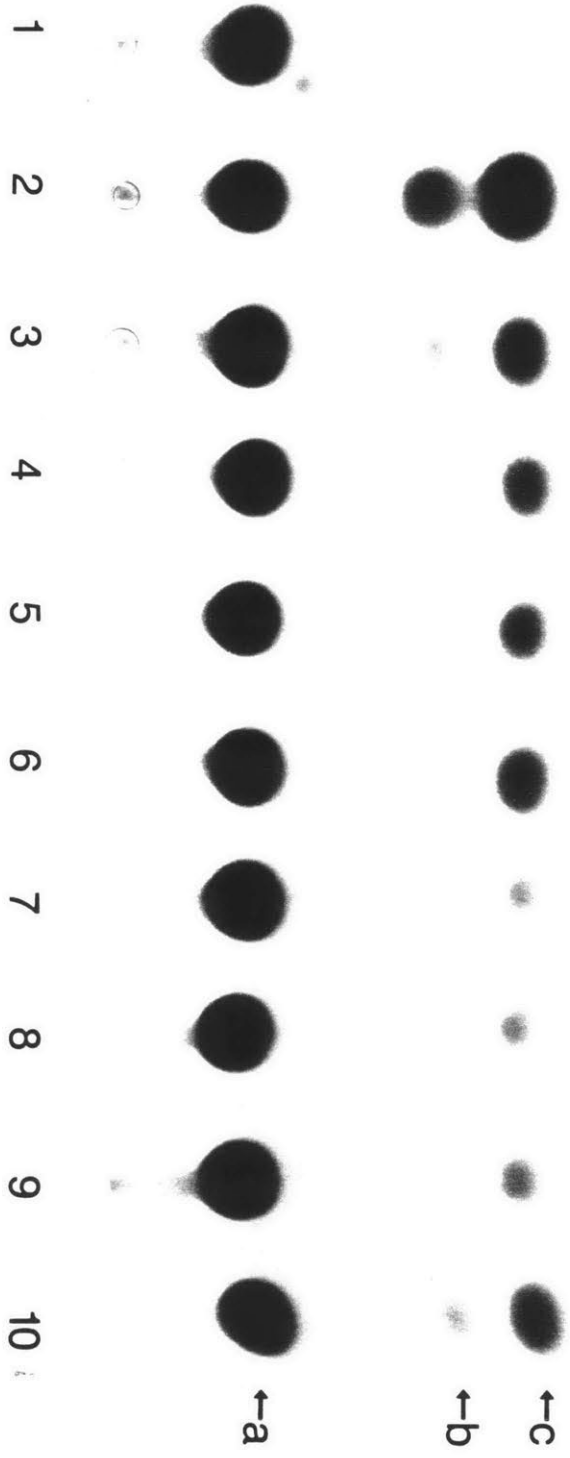
Figure HSPG: Analysis of transcription NEOHSPCAT in MEL cell line, 3a3. HSPG-A shows a typical lane of a Northern blot containing total RNA probed with any of the DNAs described in the text. HSPG-B is a Northern blot containing cytoplasmic RNA probed with CAT insert. Lane 1 represents RNA that was not heat shocked. Lanes 2-4 represents RNA that was heat shocked for 2 hours at 43^{o}C and chased for 0, 2 and 4 hours, respectively. HSPG-C is the photograph of the gel which became the Northern in HSPG-B.


A
←28s
←18s
B
1 2 3 4
C
1 2 3 4

(A)-selected on oligo(dT) cellulose and run on a gel. After reprobing with both probes, still no change in hybridization was seen. Figure HSPG-B shows the results of hybridization with purified CAT insert of a Northern blot of cytoplasmic RNA of HSPCAT 3a3 after no heat shock or after 0, 2, or 4 hours following a 2 hour heat shock. Clearly, one band is discernible, representing the CAT transcript with its 65 bases of Drosophila heat shock transcriptional leader (1.5 kb long). The broadness of the band could easily represent varying length of poly(A) tail.

Unfortunately, there was no transcriptional induction with heat shock of the heat shock driven CAT transcript in these cells. Figure HSPG-C is a photograph of the gel which was transferred and probed in Figure HSPG-B, which shows there was even loading of cytoplasmic RNA. A similar lack of transcriptional induction was obtained using mouse hsp70 gene as probe (supplied by R. Morimoto). CAT activity was assayed in these cells after no heat shock or after a one hour heat shock at 42.5^{o}C followed by a 0, 2, or 6 hour chase at 37^{o}C. As shown in Figure HSPH, there was little to no enhancement of protein activity either (lanes 3-6). A second cell line, 3b2, exhibited a similar response of CAT activity to heat, though it may be enhanced slightly at 6 hour of chase (Figure HSPH, lanes 7-10). Potential reasons for this behavior will be analyzed in the discussion.

Figure HSPH: Assay of CAT activity in two MEL cell lines harboring, NEOHSPCAT. Four flasks of each of the two stable cell lines, 3a3 (lanes 3-6) and 3b2 (lanes 7-10), were either not heat shocked (lanes 3 and 7) or heat shocked for 1 hour at 42.5^{o}C followed by 0 (lanes 4 and 8), 2 (lanes 5 and 9) or 6 (lanes 6 and 10) hours of chase at 37^{o}C. Equal aliquots (40 μl) were incubated with ^{14}C-chloramphenicol and acetyl CoA at 37^{o}C for 45 minutes. Ethyl acetate extracted samples were run on TLC. The figure represents an autoradiogram of the chromatograph in which the parent substrate and the modified forms are as marked by arrows (a is chloramphenicol, b is 1-acetate chloramphenicol, c is 3-acetate chloramphenicol). Lane 1 was incubated with extract from untransfected 3T3 cells. Lane 2 was incubated with .01 units of purified CAT enzyme, (P-L Biochemicals).


1
2
3
4
5
6
7
8
9
10
a
b
c

Discussion

These studies with the heat shock promoter were both encouraging and discouraging with respect to how worthwhile and helpful an inducible promoter would be in studying RNA stability. In 3T3 cells, the CAT assay demonstrated that the system could work. The protein activity behaved as expected, suggesting strongly that the RNA would exhibit comparable kinetics. In addition, the total RNA which hybridized to CAT sequences induced well and a determination of cytoplasmic RNA half-life could be made. In 3T3 cells into which NEOHSP123MYC and NEOHSP23MYC had been transfected, cytoplasmic RNA permitted the detection of a single RNA species which hybridized to *myc* sequences. The RNA appeared, from not being detectable in unshocked cells, and then disappeared.

Unfortunately, the data were not reproducible, in that, when the same RNA was run on two different gels, different half-lives were obtained. In addition, the half-lives obtained were quite different from the published values of 15 minutes for the entire transcript (Dani *et al.*, 1984) and greater than 50 minutes for transcripts lacking the first exon (Eick *et al.*, 1985). A possible explanation for this discrepancy is that both of the heat shock promoted *myc* messages carried 65 bases of *Drosophila* heat shock message 5' untranslated region which could account for the unexpectedly prolonged half-lives of both messages. In bacteria, it has recently been shown that even a few bases can affect the half-life of a bacterial message (K.O.Cho, personal communication). A β^0 thalassemic patient was shown to have a very short-lived β-globin messenger (Maquat *et al.*, 1981) as a result of a single base pair change at codon 60 (Kinniburgh *et al.*, 1982).

For the studies with NEOHSPCAT in MEL cells, although NEOHSPCAT was successfully introduced into these cells by protoplast fusion, no heat shock induction of transcription or translation was observed, only constitutive. Constitutive expression of hsp70 has also been observed in other erythroid cells. For example, Banerji et al. (1984) showed that hsp70 is transcribed in non-heat shocked chicken reticulocytes.

In other systems it is becoming evident that the heat shock proteins are not only stress-induced proteins, but may play a role in normal development and differentiation. Mouse hsp70 protein has been detected in two-cell mouse embryos (Bensaude et al., 1983), as well as in mouse embryonal carcinoma cells (Bensaude and Morange, 1983; Morange et al., 1984). Bienz (1984) showed that a Xenopus hsp70 gene injected into Xenopus oocytes behaves as the endogenous counterpart which actively transcribes hsp70 mRNA and stores it maternally. When the same gene was transfected into COS cells it also was transcribed constitutively as was its endogenous counterpart. For this reason the heat shock promoter may not be a useful tool for inducing a pulse of transcription in MEL cells, erythroid cells, early embyronic cells, oocytes or probably other tissues in which hsp70 is endogenously active.

In addition, heat shock itself may have an effect on the stability of specific messages. Earlier studies suggested that there was no change in the concentration of various RNAs during heat shock, but that they were translationally controlled (Ballinger and Pardue, 1983; Hickey and Weber, 1982a, 1982b; Thomas and Mathews, 1982). Wolffe et al. (1984) showed that the estrogen induced transcriptional activation and stabilization of vitellogenin mRNA in Xenopus hepatocytes is reversed by treatment with heat. Belanger et al. (1986) showed that in the aleurone cells of barley seeds a phytohormone induces the secretion of α-amylases via a set of

stable RNAs. Heat shock suppresses the synthesis of these proteins via a mechanism which concomitantly degrades their mRNAs and destroys the rough endoplasmic reticulum on which their polyribosomes translate message. Farrell-Towt and Sanders (1984) showed how heat shock affects the concentration of various histone proteins differently in Drosophila cells. Messages for histones H2a, H2b, H3 and H4 are all more stable during heat shock, than at control temperatures; however, only H2b is efficiently translated on polyribosomes. The other three are not found on polyribosomes and their transcription is diminished. The message for histone H1 is not transcribed and its message is preferentially degraded.

Heat shock has also been shown to be capable of causing proteins to unfold (Lin and Zabin, 1972; St. John et al., 1978). Such denaturation of RNases could easily modify their function and effect changes in the half-lives of certain or all RNAs. In light of observations showing heat shock to be non-inducible in many systems and to have differential effects on the half-lives of messages, the use of the heat shock promoter to study RNA stability has significant limitations.

Figure HSPA: Construction of NEOHSPCAT from PMSVNEO and HSPCAT-1. Pertinent sites are as marked on the plasmids. Arrow a : PMSVNEO was cut with Sal1 and treated with CIP (Calf Intestine Phosphatase). Arrow b: HSPCAT-1 was cut with Pvu1 and BamH1. 4.5 kb piece containing HSPCAT was blunted by treatment with Klenow fragment of DNA polymerase I. Sal1 linkers were ligated on at 50-fold molar excess and cut off. Arrow c: two fragments were ligated at 20 μg/ml transformed into DH-1 bacteria and plated onto L-B agar with 50 μg/ml ampicillin and 50 μg/ml kanamycin. Arched arrows indicate the direction and extent of transcription. Arched lines indicate the source of DNA in the plasmid.

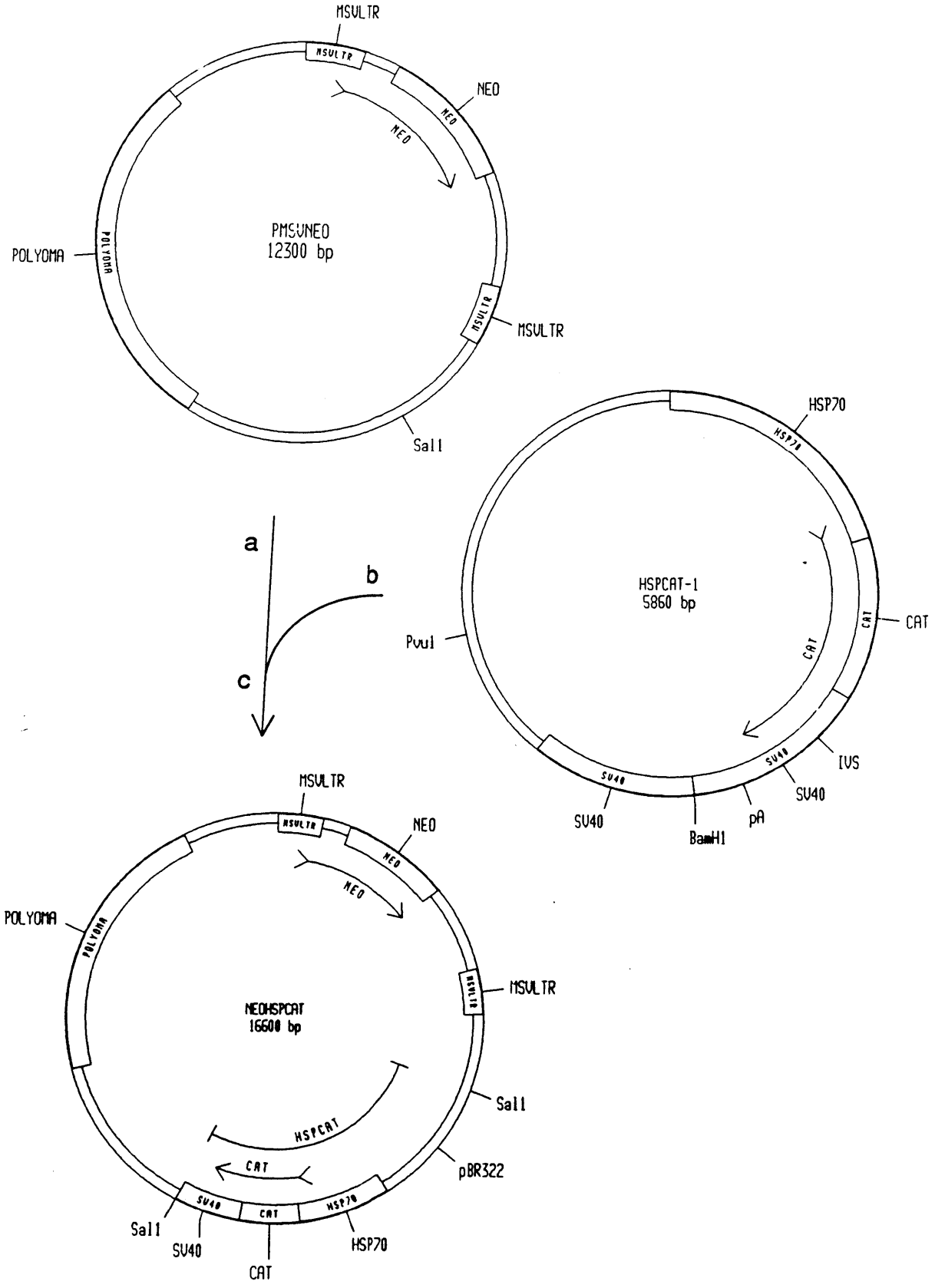

MSVLTR
NEO
PMSVNEO
12300 bp
POLYOMA
MSVLTR
SalI
a
b
c
HSP70
HSPCAT-1
5860 bp
CAT
PvuI
IVS
SV40
SV40
pA
SV40
BamHI
MSVLTR
NEO
POLYOMA
NEOHSPCAT
16600 bp
MSVLTR
SalI
pBR322
HSPCAT
CAT
SV40
CAT
HSP70
SalI
SV40
CAT
HSP70

Figure HSPC: Construction of PSV2NEOHSP from PPW229 and PSV2NEO. Pertinent sites are as marked on the plasmids. Arrow a: PSV2NEO was cut with BamH1 and treated with CIP (Calf Intestinal Phosphatase). Arrow b: PPW229 (Livak et al., 1987) was cut with BamH1 and the 2.6 kb piece containing the Drosophila hsp70 promoter was isolated. Arrow c: two fragments were ligated at 20 μg/ml, transformed into HB101 bacteria and plated onto L-B agar containing 50 μg/ml ampicillin. Arched arrows indicate the direction and extent of transcription. Arched arrows indicate the source of DNA in the plasmid.

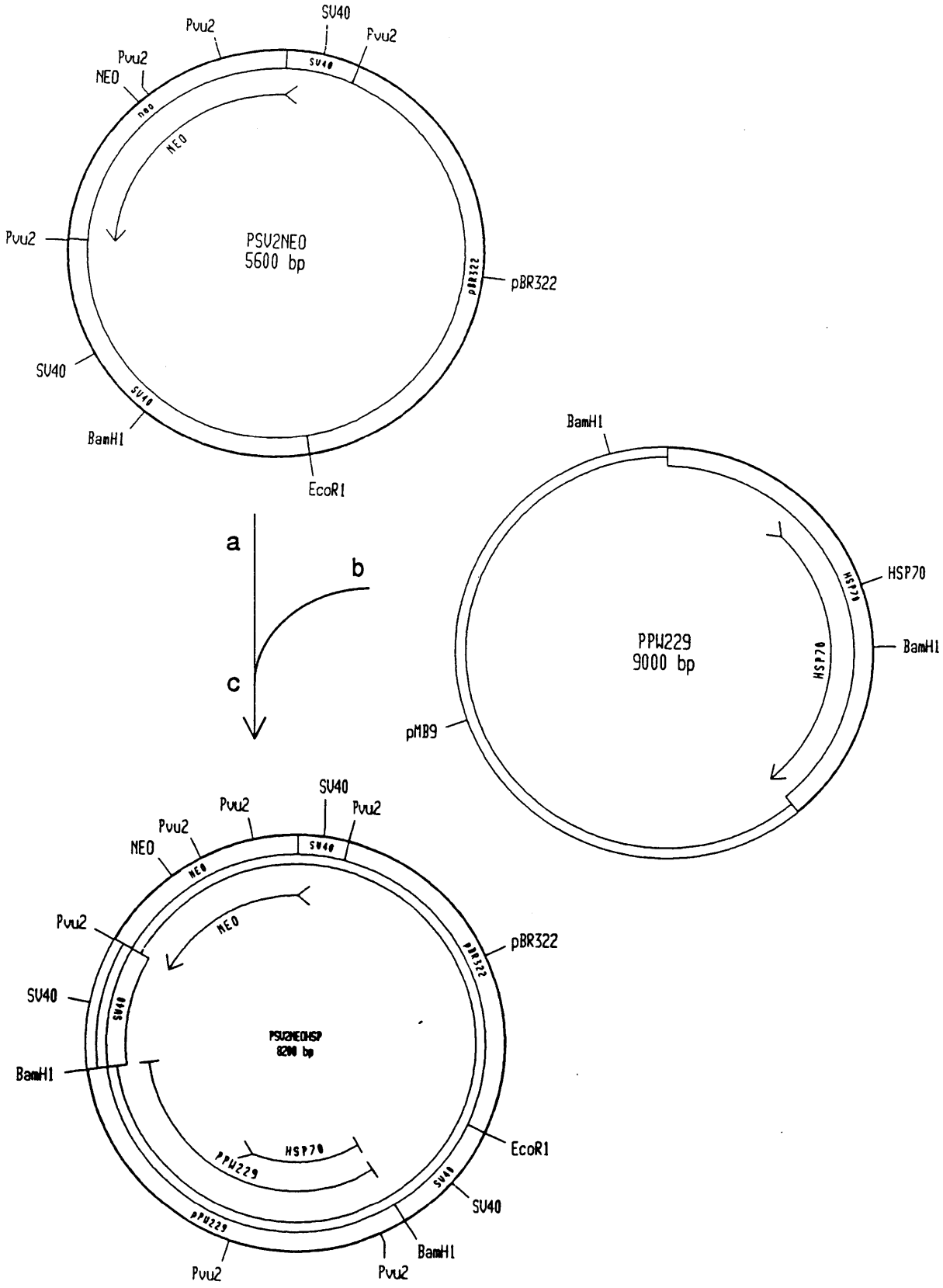

SV40
Pvu2
Pvu2
NEO
Pvu2
PSV2NEO
5600 bp
pBR322
SV40
BamH1
EcoR1
a
b
c
BamH1
HSP70
PPW229
9000 bp
BamH1
pMB9
SV40
Pvu2
Pvu2
NEO
Pvu2
pBR322
SV40
BamH1
EcoR1
SV40
BamH1
Pvu2
Pvu2

Figure HSPD: Construction of NEOHSP123MYC from PSV2NEOHSP AND HUCMYC: Pertinent sites are as marked on the plasmids. Arrow a: PSV2NEOHSP was cut partially in the presence of 65 μg/ml ethidium bromide with .5 u/μg plasmid DNA of Pvu2 for 30 minutes. (The concentration of ethidium bromide used was determined by its titration from 0 to 200 μg/ml under similar conditions to maximize for single cut plasmids (Parker et al., 1976).) Linear, single cut DNA was isolated from a gel and recut to completion with EcoR1. 6.23 kb Pvu2 to EcoR1 fragment containing NEO and the hsp70 promoter to exactly the junction used in HSPCAT-1 and its derivative plasmids was isolated from the gel. Arrow b: HUCMYC was cut with Xho1, blunted by treatment with Klenow fragmet of DNA polymerase I and recut with EcoR1. The 5.5 kb fragment was gel isolated. Arrow c: Two fragments were ligated at 20 μg/ml for 3 hours at 15°C to append the sticky, EcoR1 ends. Reactions were then diluted 10-fold and incubated overnight at 30°C to allow blunt ends to ligate. The ligation was then transformed into HB101 bacteria and plated onto L-B agar with 50 μg/ml ampicillin. Arched arrows indicate the extent of transcription. Arched lines demarcate the origin of different regions of DNA in the plasmid.

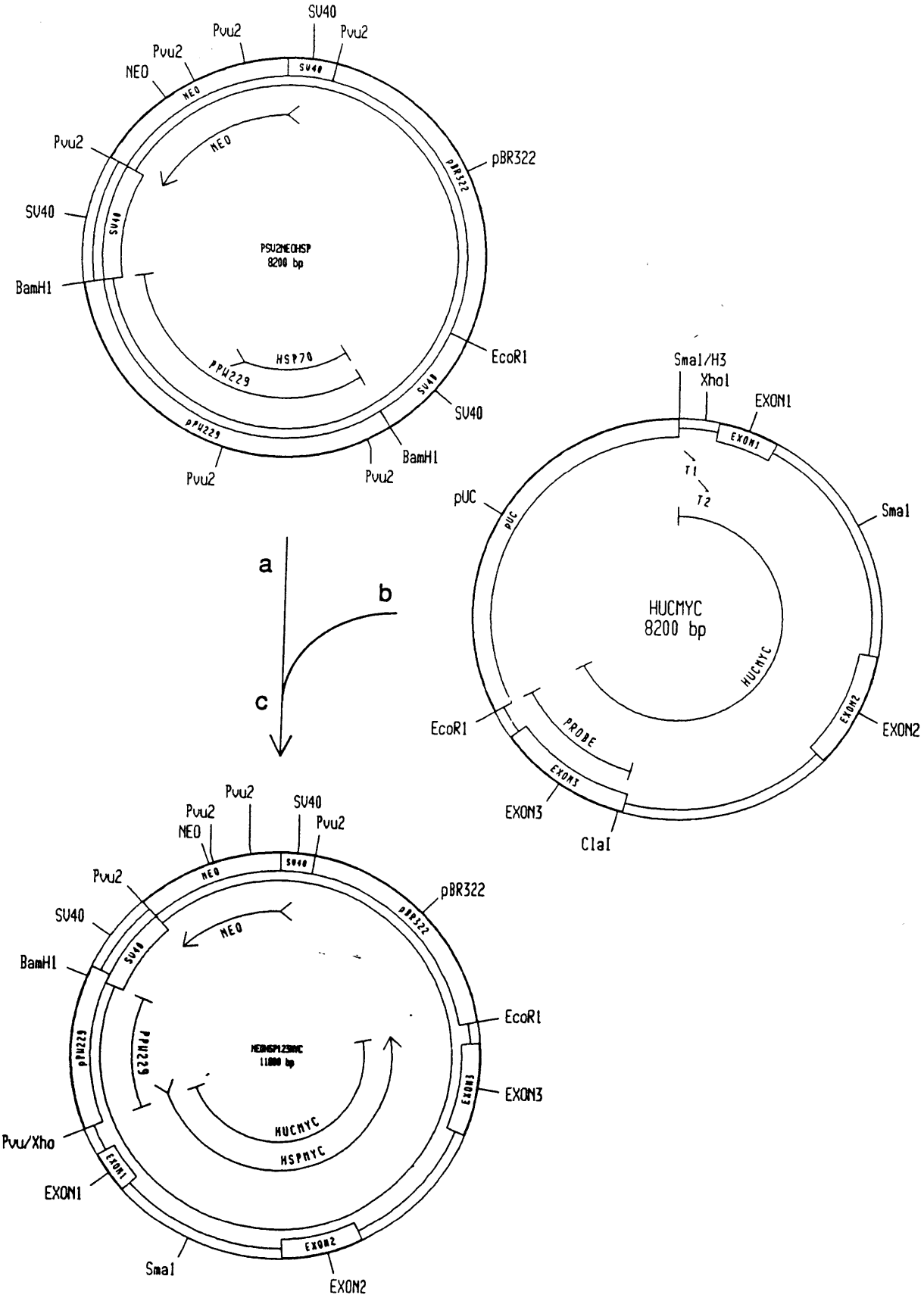
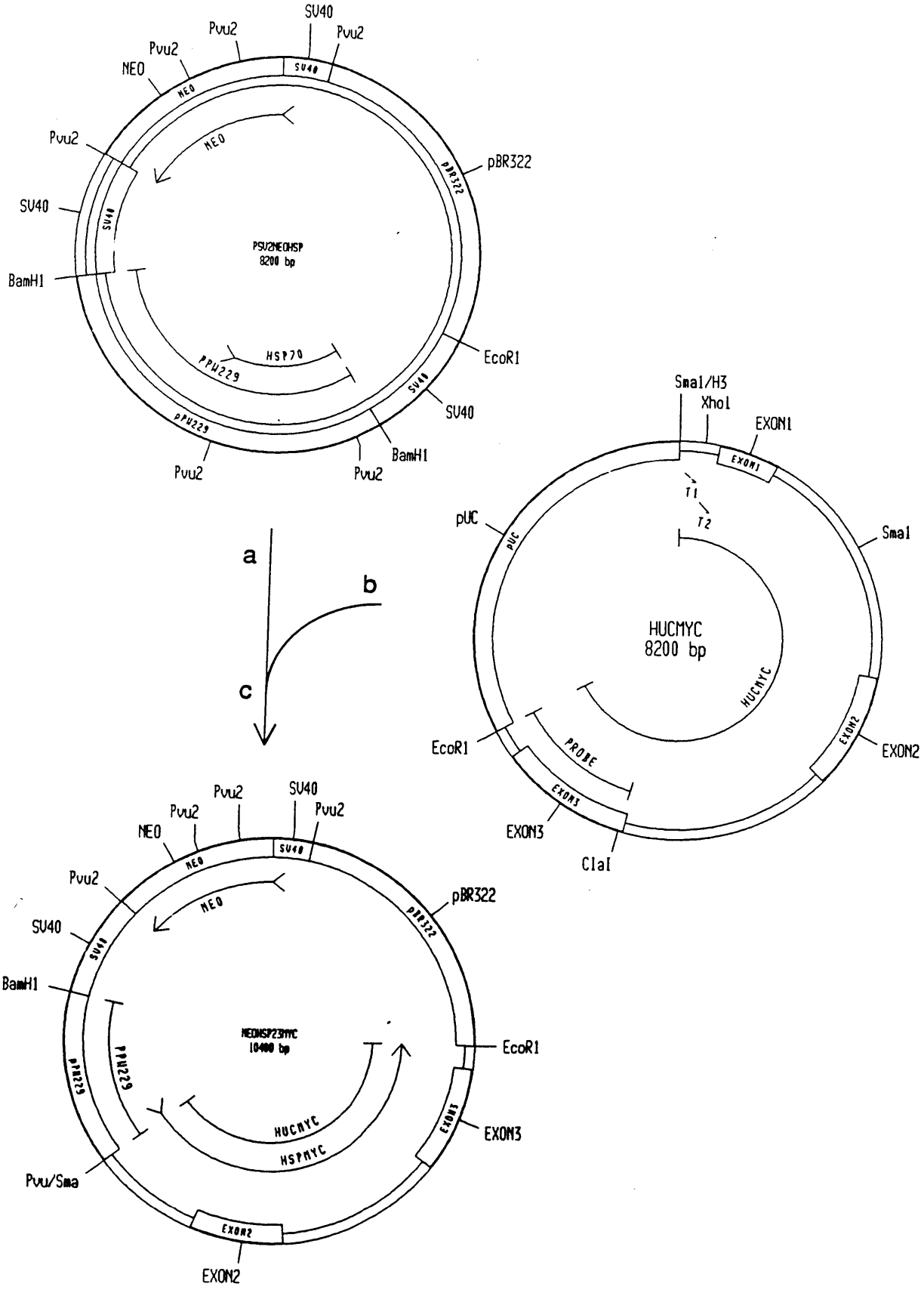

SV40
Pvu2
Pvu2
Pvu2
NEO
pBR322
Pvu2
SV40
PSV2NEOHSP
8200 bp
BamH1
HSP70
PPU229
EcoR1
SV40
BamH1
Pvu2
Pvu2
a
b
c
Sma1/H3
Xho1
EXON1
T1
T2
pUC
Sma1
HUCMYC
8200 bp
HUCMYC
EcoR1
PROBE
EXON2
EXON2
EXON3
EXON3
ClaI
Pvu2
Pvu2
SV40
Pvu2
NEO
pBR322
Pvu2
SV40
NEO
BamH1
EcoR1
PPU229
EXON3
Pvu/Xho
HUCMYC
HSPMYC
EXON1
Sma1
EXON2

Figure HSPE: Construction of NEOHSP23MYC from PSV2NEOHSP and HUCMYC: Pertinent sites are as marked on the plasmids. Arrow a: PSV2NEOHSP was manipulated as in the legend to Figure HSPD. Arrow b: HUCMYC was cut Sma1 and then recut with EcoR1 and the 4.1 kb fragment was gel isolated. Arrow c: Ligation was identical to that described in Figure HSPD. Arched arrows indicate the extent of transcription. Arched lines demarcate the origin of different regions of DNA in the plasmid.


SV40
Pvu2
Pvu2
Pvu2
NEO
NEO
SV40
Pvu2
pBR322
SV40
PSV2NEOHSP
8200 bp
BamH1
HSP70
PPH229
EcoR1
SV40
SV40
PPH229
BamH1
Pvu2
Pvu2
a
b
c
Sma1/H3
Xho1
EXON1
T1
T2
pUC
Sma1
HUCMYC
8200 bp
EcoR1
PROBE
EXON2
EXON3
EXON3
ClaI
SV40
Pvu2
Pvu2
Pvu2
NEO
NEO
SV40
Pvu2
pBR322
SV40
NEOHSP23HYC
10400 bp
BamH1
PPH229
EcoR1
PPH229
HUCMYC
HSPMYC
EXON3
Pvu/Sma
EXON2
EXON2

Chapter III

Use of the Metallothionein Promoter

Introduction

The metallothionein promoter was evaluated for its capacity to create a pulse of transcription in the experiments described in this chapter. This promoter has been well defined with respect to the sequences that are responsible for its activity. The mouse metallothionein-I (MT-I) gene promoter and a series of associated deletion mutants, all driving the herpes virus thymidine kinase gene, were microinjected into mouse egg cells (Brinster et al., 1982). These studies revealed that all sequences crucial for induction of this promoter were located within 90 bases of the transcriptional start. Karin et al. (1984b) showed with a similar series of deletion mutants that the human MT-II_A promoter retains full inducibility with Cd^{++} ions with only 50 base pairs 5' to its transcriptional start. Three metallothionein promoter regions (human MT-I_A, human MT-II_A, and mouse MT-I) had been compared, revealing a conserved GC-rich region containing a potential hairpin-loop-forming structure in addition to conserved residues from -43 to -67. These two conserved structures are reminiscent of those observed among heat shock genes. (See Chapter IIA Introduction to the Heat Shock Promoter.)

The specific promoter that was chosen was the human metallothionein II_A promoter. This endogenous gene in human skin fibroblasts is maximally induced by dexamethasone (a potent, synthetic glucocorticoid), Zn^{++}, and Cd^{++}, whereas its counterpart, metallothionein I_A, is maximally induced only by Cd^{++} (Richards et al., 1984). Since treatment of cells with cadmium ions is more toxic to cells than zinc, MT-II_A was selected. Karin et al., (1984a) had previously shown that this promoter induced well in response to Cd^{++} and dexamethasone in the heterologous Rat 2 cell line while driving the heterologous herpes simplex virus thymidine kinase gene.

Since glucorticoid induction had been avoided as described in the Chapter IE1 of the Introduction, $ZnCl_2$ was chosen as the agent for induction.

Results

A construct consisting of the neo^r gene, and the human β-globin gene driven by a human metallothionein IIA promoter (NEOMTGLO, Figure MTC) was constructed via intermediates (Figures MTA, MTB and MTC). (Figures of constructs are located at the end of each chapter.) The transcript contained 75 bp of metallothionein promoter transcriptional leader. The plasmid (10 μg) was transfected into NIH/3T3 cells (5 tissue culture plates, via Method A described in Material and Methods), and selected in G418. 159 cell lines putatively harboring this plasmid were grown up to be frozen and screened for production of the metallothionein-driven globin message. Each plate was treated with 200 μM $ZnCl_2$ for 11 hours, at which time all of the cells were floating, not attached to the surface of the dish. The cells were checked for viability using trypan blue exclusion and found to be deceased. Apparently the inducer is extremely toxic in these cells, thus further experiments with 3T3 cells were not conducted.

The construct NEOMTGLO was next tried in MEL cells. NEOMTGLO was successfully introduced into MEL cells by protoplast fusion. Of 54 G418 colonies from 8 separate tissue culture flasks, 27 were cloned out and grown up for screening. Cells were either frozen for later use, or screened for the production of the metallothionein-driven globin message. Each cell line was treated with 200 μM $ZnCl_2$ for 8-10 hours and tested for production of the metallothionein-driven globin message using an SP6 RNase protection assay using total cellular RNA. These cells were tested for viability using trypan blue exclusion and were shown to be alive. Only 2 of the 27 cell lines tested did not produce globin message. Two positive lines, C3B4 and C1B3, were tested for induction with and without $ZnCl_2$, both in the presence of normal serum and in the presence of serum that had

been dialyzed to remove any heavy metals and androgens (Figure MTD). Both heavy metals and androgens could increase the basal level of the promoter. The two cell lines showed many-fold induction of β-globin RNA in the presence of $ZnCl_2$. Both the stimulated and unstimulated levels of metallothionein-promoted RNA were considerably higher in the two cell lines when the cells were grown and treated in media containing dialyzed fetal calf serum. Apparently, a low molecular weight inhibitor molecule was dialyzed out.

A time course experiment was performed on each MEL cell line harboring NEOMTGLO that had induced metallothionein-driven message in response to treatment with $ZnCl_2$. Slot blot analyses were performed on cytoplasmic RNAs that had not been treated or that had been treated with 200 μM $ZnCl_2$ for 8.5 hours and then chased for 3, 6, 10.5, 15.5, 28, and 53 hours in the absence of $ZnCl_2$. The slot blots were normalized and quantitated as described in the Appendix. Both slot blots gave statistically acceptable data with an average half-life of 57 minutes (See Table NEOMTGLO). However, this value is much shorter than expected (see below) and concern over the possible toxic effects of the inducer on RNA half-lives in these cells caused us to no longer pursue this approach with this promoter.

Figure MTD: Slot blot analysis of MEL cell lines harboring NEOMTGLO induced with $ZnCl_2$, in the presence of normal serum and in the presence of serum dialyzed to remove heavy metal ions and steroids. Columns A, B, and C have 15 μg, 7.5 μg and 3 μg, of total RNA, respectively. Rows 1-4 contain RNA from cell line C3B4, rows 5-8 contain RNA from cell line C1B4. Rows 1, 2, 5, and 6 contain RNAs from cells grown and induced in the presence of normal serum. Rows 3, 4, 7, and 8 contain RNAs cells grown in the presence of dialyzed serum. Rows 1, 3, 5, and 7 contain RNAs from cells treated with 200 μM $ZnCl_2$ for 9 hours. Rows 2, 4, 6, and 8 contain RNAs from cells not treated with $ZnCl_2$. Total RNA was incubated for 15 minutes at 65^{o}C with 3 volumes of 6.15 M formaldehyde and 10X SSCPE. Samples were transferred to ice and loaded onto Gene Screen Plus (NEN) via Schleicher and Schuell Slot Blot II Apparatus according to the manufacturer's instructions. The blot was probed with 2.6 kb human β-globin probe.

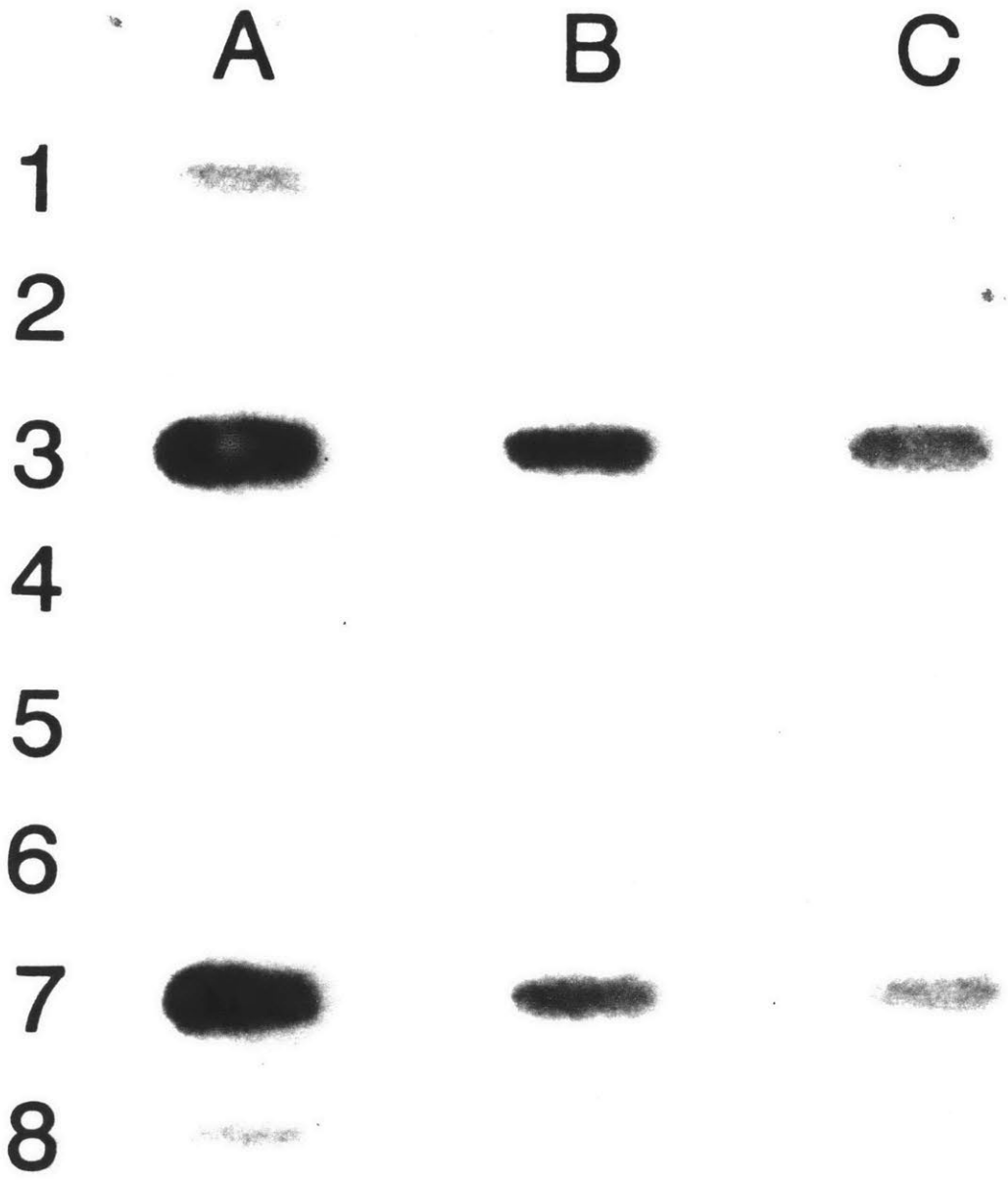

A
B
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7
8

Table MT

NEOMTGLO

CELL LINE	EXPT.	BLOT	1/2-LIFE±S.D.[5]	t[1]	D.F.[2]	P[3]	AVERAGE±S.D.[4,5]
C1B4	8/2/85	8/5/85	36±6	6.0	2	.025<P<.05	
C3B4	8/2/85	8/5/85	78±12	6.5	3	.005<P<.01	57±30

1 t-statistic (See Appendix)
2 degrees of freedom (See Appendix)
3 P-value ≤ .05 is acceptable (SeeAppendix)
4 average of statistically acceptable half-lives
5 half-lives in minutes

Discussion

Mayo and Palmiter showed that Cd^{++} induced endogenous mouse MT-I mRNA over 13-fold in 3T3 cells and over 6-fold in MEL cells (Mayo and Palmiter, 1981). This observation implied that (1) the endogenous protein was capable of protecting the cells from metal poisoning, and (2) a heterologous gene might also be induced to a sufficient extent. In addition, transfection of the human MT-II_A gene enabled NIH/3T3 cells to survive in inducing concentrations of Cd^{++} (Richards et al., 1984). In our experiments, however, 3T3 cells were not protected from heavy metal distress by their endogenous metallothionein proteins. A titration of metal might have revealed a concentration at which both exogenous and endogenous promoters could be induced and enough endogenous protein would be made to safeguard the cell since the endogenous gene does induce and could probably protect the cell from low concentrations of metal. In addition, the entire human MT-II_A gene could have been cotransfected with the fusion gene (human MT-II_A promoter and human β-globin gene) to behave as a selectable marker.

MEL cells, however, were able to protect themselves from metal poisoning. Inducible MEL cell lines were obtained by protoplast fusion of NEOMTGLO. Both cell lines gave statistically acceptable, but different, half-lives for the fusion MT-II_A-globin transcript, 36 and 78 minutes (average 57 minutes). These values are much shorter than the expected half-life based on the data supplied in Table Introduction B which would suggest the half-life should be between 17 and 60 hours. This unexpectedly short half-life could be due to the 75 bp transcriptional leader (as described in Chapter II on the Heat Shock Promoter) or the result of toxic effects of the zinc treatment used for inducing the promoter. Treatment

with heavy metals can potentially alter the half-lives of RNAs simply by stressing the cells as was shown with the heat shock promoter. Heavy metals have been shown to bind to sulfhydryl groups and effect changes in the conformation of proteins (Fluharty and Sanadi, 1960; Levinson et al., 1980). Such alterations in RNase structure could easily modify the function of such a protein resulting in altered half-lives of messages. Because of these issues associated with heavy metal toxicity we do not find the metallothionein system to be attractive for the study of mRNA stability.

Figure MTA: Construction of NEOMT. Pertinent sites are marked on the plasmids. Arrow a: PUCMT (pHS1 from A. Haslinger and M.Karin; Karin and Richards, 1982) was cut with Hind3, blunted with Klenow fragment and then recut with EcoR1. The .85 kb fragment containing the human metallothionein II_A promoter (hMTIIA) was isolated on a gel. Arrow b: PSV2NEO was cut with BamH1, blunted with Klenow fragment and recut with EcoR1. The 4.85 kb fragment containing NEO and vector sequences was gel isolated. Arrow c: Two fragments were ligated for 3 hours at 15°C to allow the EcoR1 ends to anneal and ligate. The reaction was diluted 10-fold and incubated overnight at 30°C in order to circularize the blunted ends. Ligation was transformed into HB101 and plated onto L-B agar containing 50 μg/ml ampicillin. Arched arrows indicate the extent of transcription.

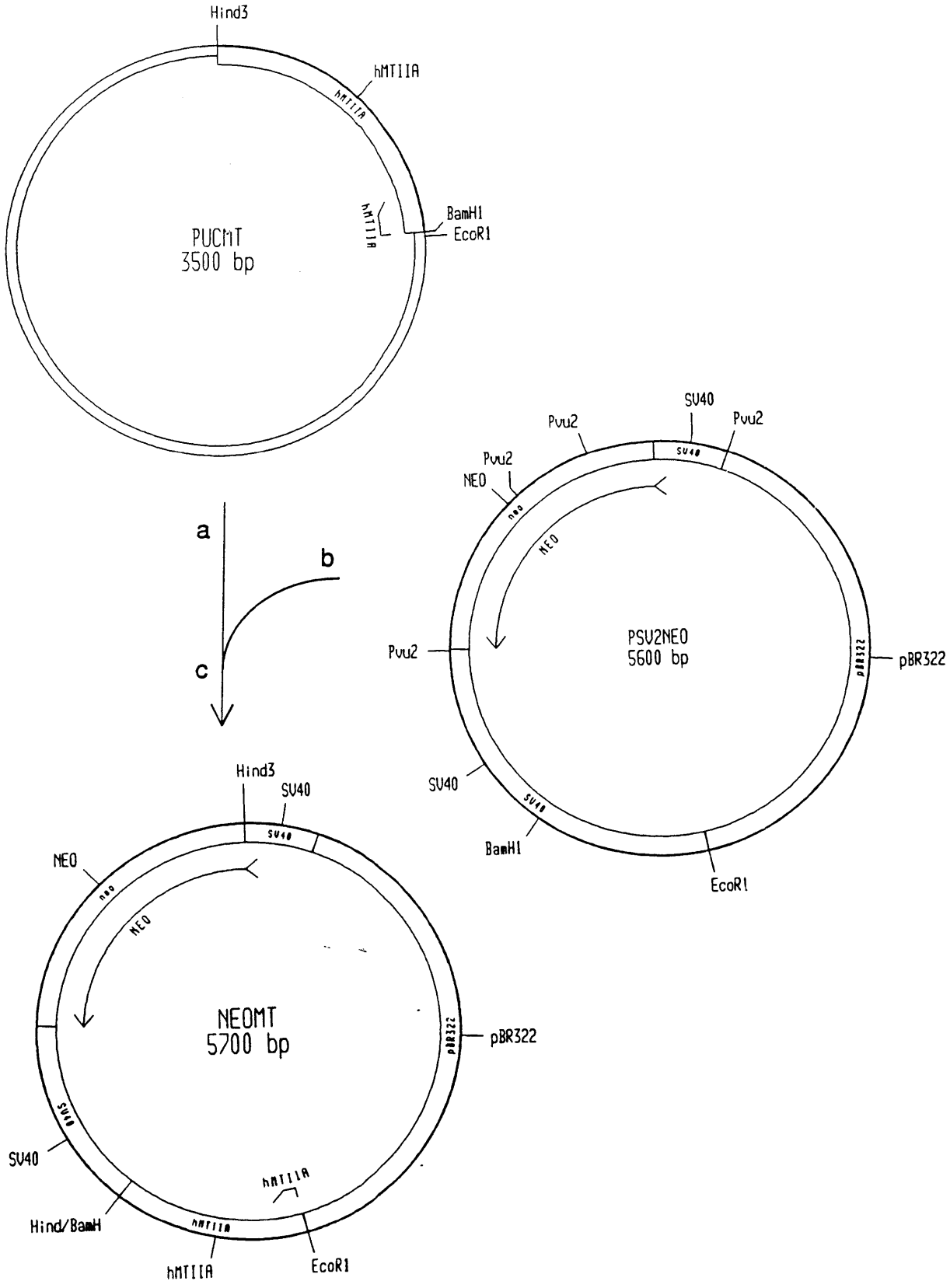

Hind3
hMTIIA
BamH1
EcoR1
PUCMT
3500 bp
a
b
c
SV40
Pvu2
Pvu2
Pvu2
NEO
PSV2NEO
5600 bp
pBR322
Pvu2
SV40
BamH1
EcoR1
Hind3
SV40
NEO
NEOMT
5700 bp
pBR322
SV40
Hind/BamH
hMTIIA
hMTIIA
EcoR1

Figure MTB: Construction of NEOMTGLOP, an intermediate in the construction of NEOMTGLO. Pertinent sites are as indicated on the plasmids. The sections marked "coding" stretch from the cap to the end of the message and include introns. Arrow a: NEOMT was cut with BamH1, blunted with Klenow fragment and recut with EcoR1. The 5.7 kb DNA was gel purified. Arrow B: H3HUBGLO (obtained from R. Treisman as it contained an inserted Hind3 site right upstream of the GLO capping site, Treisman et al., 1983) was cut with Hind3, blunted with Klenow fragment and recut with EcoR1. The 1.5 kb piece from Hind3 to EcoR1A containing the 5' half of GLO was gel purified. Arrow c: The two fragments were ligated and transformed as described in the legend to Figure MTA. Arched arrows indicate the extent of transcription. Arched lines indicate the source of the DNA in the plasmid.

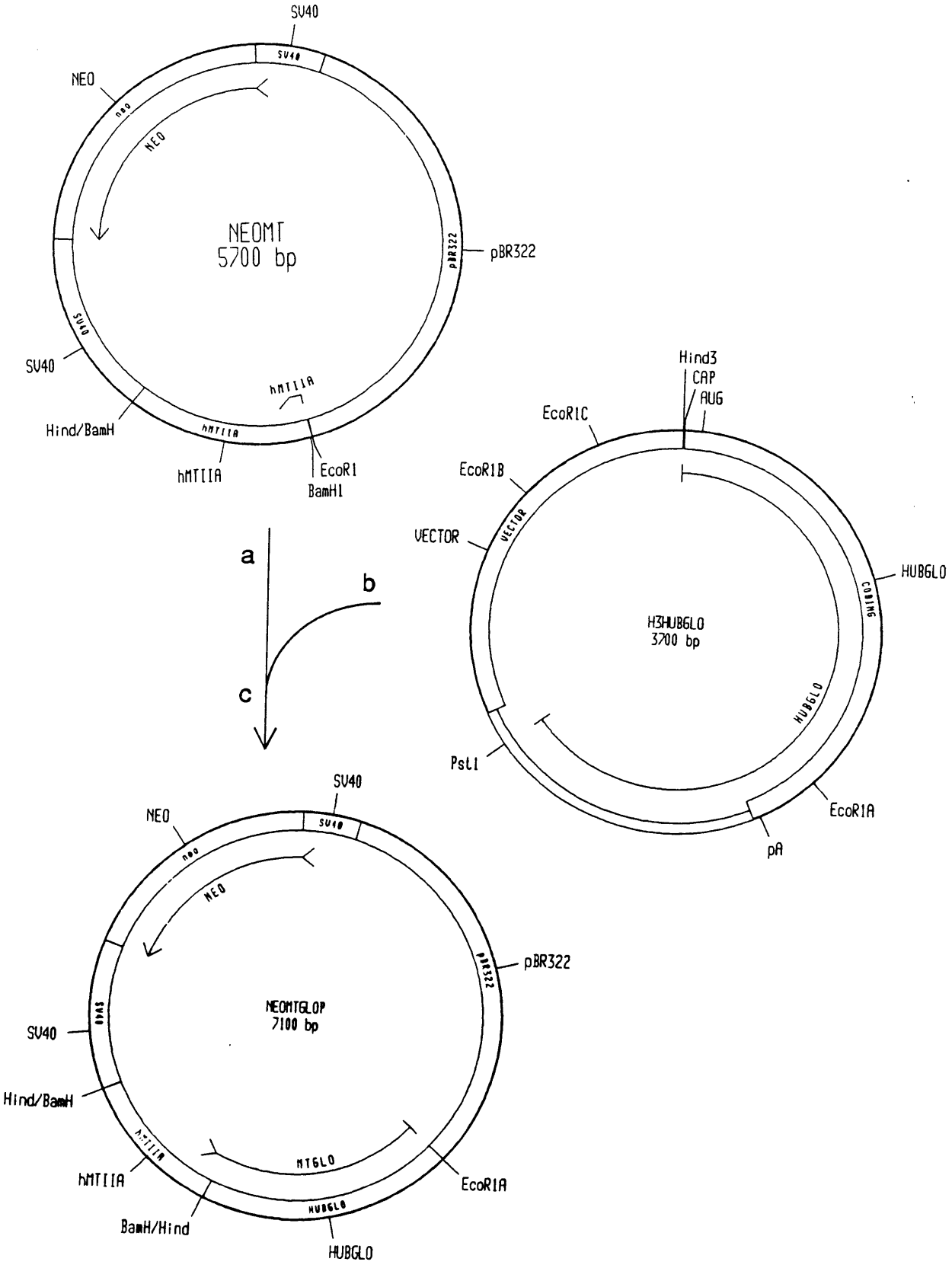
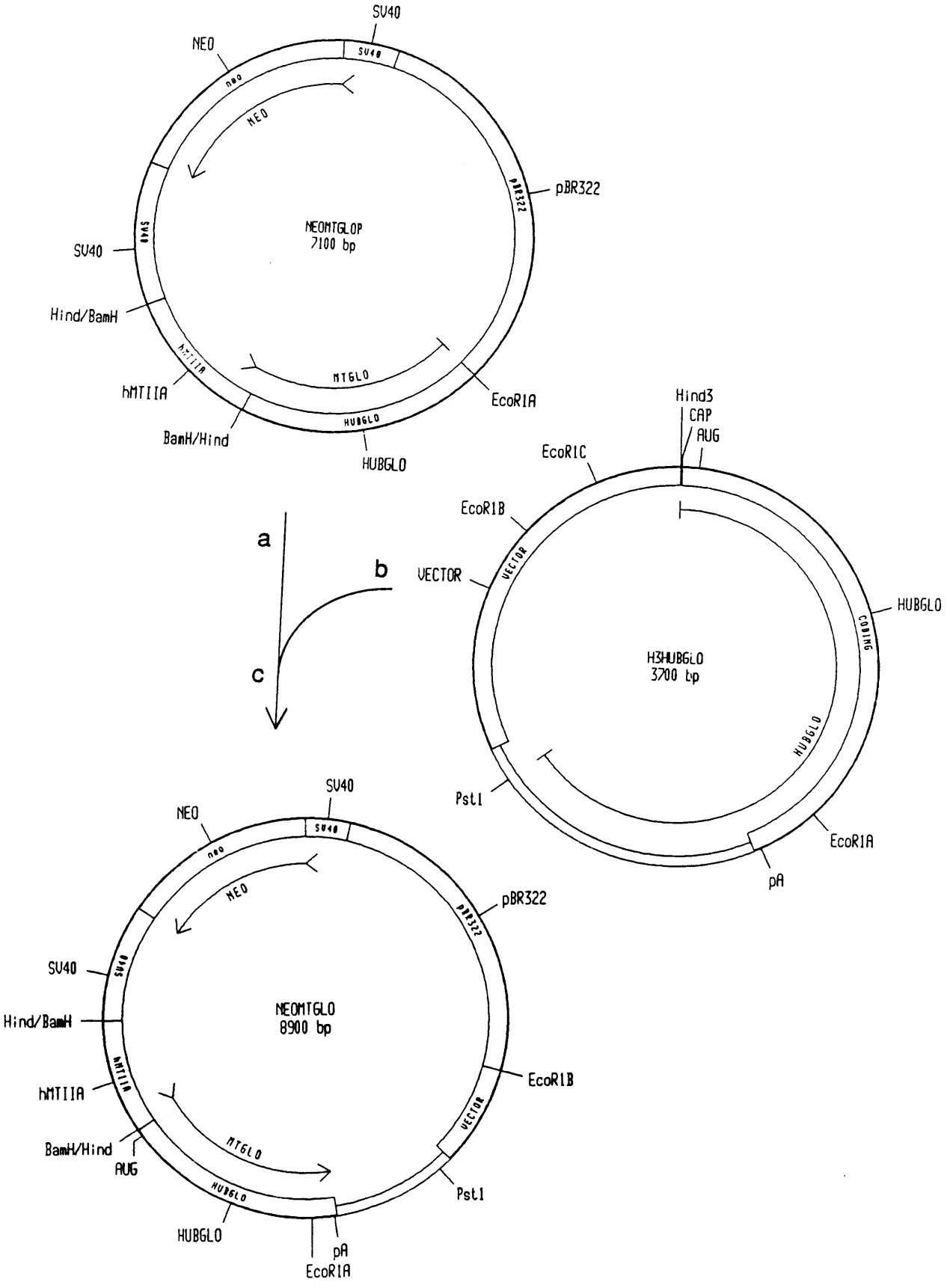

SV40
NEO
NEO
NEOMT
5700 bp
pBR322
SV40
SV40
hMTIIA
Hind/BamH
hMTIIA
hMTIIA
EcoR1
BamH1
a
b
c
Hind3
CAP
AUG
EcoR1C
EcoR1B
VECTOR
VECTOR
HUB6LO
CODING
H3HUB6LO
3700 bp
HUB6LO
PstI
EcoR1A
pA
SV40
NEO
NEO
pBR322
NEOMTGLOP
7100 bp
SV40
Hind/BamH
hMTIIA
MTGLO
EcoR1A
BamH/Hind
HUBGLO
HUBGLO

Figure MTC: Construction of NEOMTGLO. Pertinent sites are marked on the plasmids. The section marked coding stretches from the cap to the end of the message and includes introns. Arrow a: NEOMTGLOP was cut with EcoR1 and treated with CIP (calf intestine phosphotase) to prevent religation of the ends. Arrow b: H3HUBLGO was also cut with EcoR1, and the 1.8 kb fragment from EcoR1A to EcoR1B containing the 3' half of GLO was gel purified. Arrow c: The two fragments were ligated for 3 hours at 15^{o}C and then transformed as described in the legend to Figure MTA. Arched arrows indicate the extent of transcription. Arched lines indicate the source of the DNA in the plasmid.


SV40
NEO
pBR322
NEOMTGLOP
7100 bp
SV40
Hind/BamH
hMTIIA
BamH/Hind
HUBGLO
EcoR1A
MTGLO
a
b
c
Hind3
CAP
AUG
EcoR1C
EcoR1B
VECTOR
HUBGLO
H3HUB6LO
3700 bp
Pst1
EcoR1A
pA
SV40
NEO
pBR322
NEOMTGLO
8900 bp
SV40
Hind/BamH
hMTIIA
BamH/Hind
AUG
HUBGLO
pA
EcoR1A
EcoR1B
VECTOR
Pst1
MTGLO

Chapter IV

Use of the Fos Promoter

Introduction

The final attempt at using an inducible promoter to study mRNA stability involved the use of the promoter of the human c-fos gene as suggested by B.H. Cochran. The promoter is experimentally activated by treating cells with media containing 15% calf serum following a one to three day period of growth arrest in media containing 5% platelet-poor plasma, which is deficient in growth factors.

Studies have identified regions 5' to the human c-fos gene which are responsible for transcriptional induction by serum factors (Treisman, 1986). A 22 bp synthetic copy of the symmetrical sequence located 300 bp upstream of the fos capping site competed specifically for protein binding and was able to confer serum inducibility on human c-fos genes that lacked this activating element. The constructs used in our experiments all contain roughly 700 bp of 5' DNA upstream of the fos transcriptional start site.

Other characteristics of the human c-fos gene were attractive in this system. The message is highly, rapidly and transiently inducible. In quiescent mouse fibroblasts, Muller et al.,(1984) showed that endogenous c-fos message levels rose dramatically by several orders of magnitude with one hour of serum-stimulation. By the end of the next hour, the RNA had returned to basal levels. The system would only be useful if large amounts of fos-driven transcripts could be produced, as this would facilitate detection and quantitation of decay. The fact that the message appeared so rapidly, in such massive quantities, and then disappeared so rapidly was important for two reasons. First, it implied that the promoter reacted immediately to induction and that it turned off as quickly. A brief, strong pulse of transcription was required for this system to facilitate the study of the decay of that pulse. A promoter whose transcription does

not turn off rapidly could give an artificially prolonged message half-life, which may have been part of the problem with the heat shock promoter. In the event that RNA synthesis were not shut off rapidly one would predict that the log of the concentration of the message would not show a linear decay with respect to time. Second, the rapid disappearence of fos message suggests that it is very short-lived and would, therefore, be informative in studies of fos-globin chimeras since they have such disparate half-lives. The half-life of the fos message has been documented to be 9 minutes (Rahmsdorf et el.,1987) compared with the much longer life of globin (see Table Introduction B). The intention in constructing chimeras was to identify sequences which could be responsible for signalling to the cell's degradative system the rate at which a given species should be destroyed.

Our initial goal was to determine if the human c-fos promoter could be effectively induced in heterologous cells while driving the transcription of other mRNA species and maintaining the characteristics described above. Chimeric DNAs were constructed such that the junction sites did not disrupt the coding sequences and roughly divided the transcripts into 5' untranslated, coding, and 3' untranslated regions. Half-lives for a series of fos promoter-driven constructs have been obtained and are discussed.

Results

Preparation of Plasmids:

All plasmids were constructed without the neo^r gene, as cotransfection with the selectable marker is possible in 3T3 cells (Southern and Berg, 1982). Figures FOSA-FOSL (located at the end of this chapter) illustrate the construction of the chimeras between human c-fos and human β-globin, as well as all intermediate plasmids that facilitated the construction of the final plasmids. The details of the constructs are described in the figure legends. Within a construct the length of an arc represents the relative amount of that DNA in that plasmid. GLO5 was constructed in order to obtain the "perfect" globin message, one which used the globin capping site, driven from the fos promoter. It was essential to ensure that a given half-life was due to the structure of the message and not any extra bases from the transcriptional leader of the promoter. The construct was made via multiple intermediates, including an M13 fos-glo junction that was created using an oligomer. The fos-glo promoter-message junction was sequenced and shown to be exactly as it had been designed: the junction site was 4 bases upstream of the globin capping site and approximately 4 bases upstream of the fos capping site (Figure FOSG). GLOFOS7, GLOFOS8, and GLO9 are all derived from GLO5, so they also use the proper globin capping site. Both human c-fos and human β-globin DNAs are genomic in origin (Curran et al., 1983; Miller et al., 1984; Fritsch et al., 1979). A comparative schematic representation of genomic counterparts of each message driven from the fos promoter, drawn to scale, is given in Figures FOSM and FOSN.

A section of globin, a 2.6 kb piece of genomic DNA from Nco1 to Pst1, was used as probe for constructs FOSGLO3, FOSGLO4, GLO5, GLOFOS8, and GLO9. To distinguish exogenous human c-fos RNA from endogenous mouse c-fos RNA, a

Figure FOSM: Genomic, unspliced versions of human c-fos, GLO5, FOSGLO3, and FOSGLO4. Thick lines represent fos 5' and 3' untranslated regions and intervening sequences. Open blocks represent fos protein coding regions. Thin lines represent globin 5' and 3' untranslated regions and introns. Hatched blocks represent globin protein coding sequences. The scale is shown.

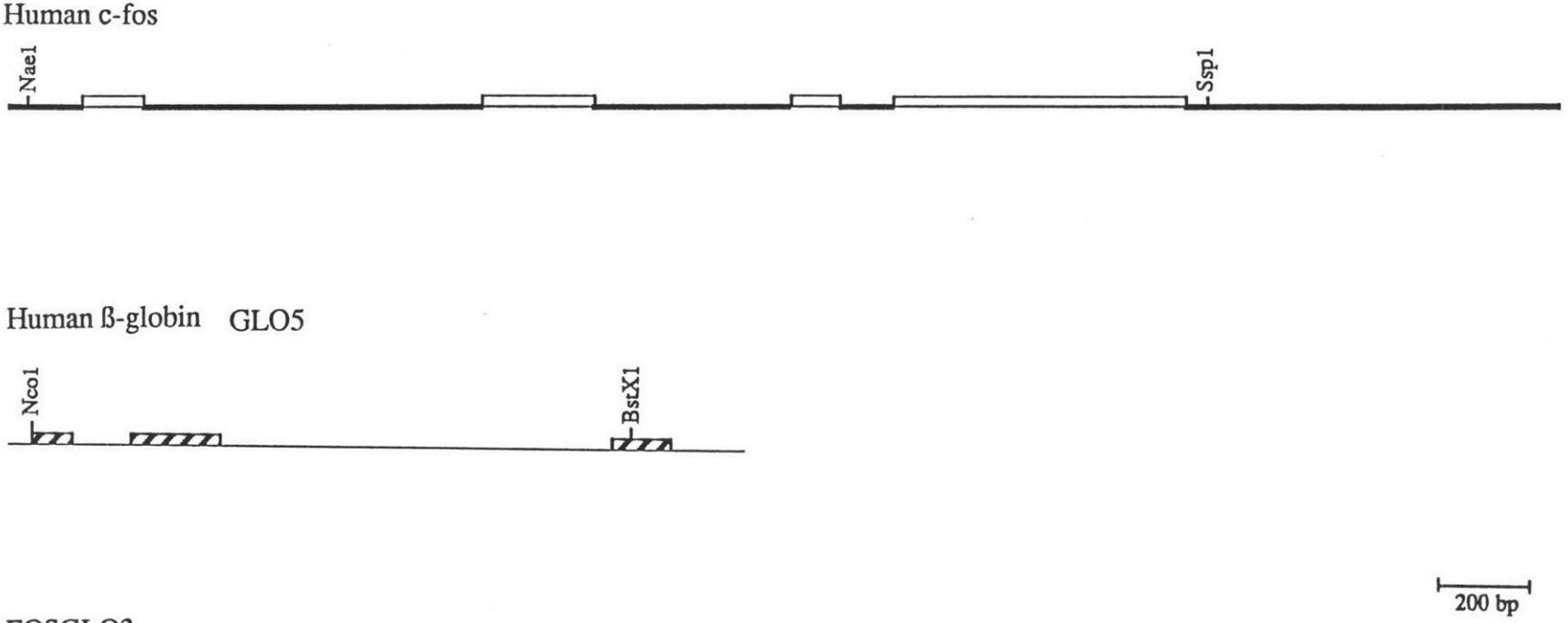
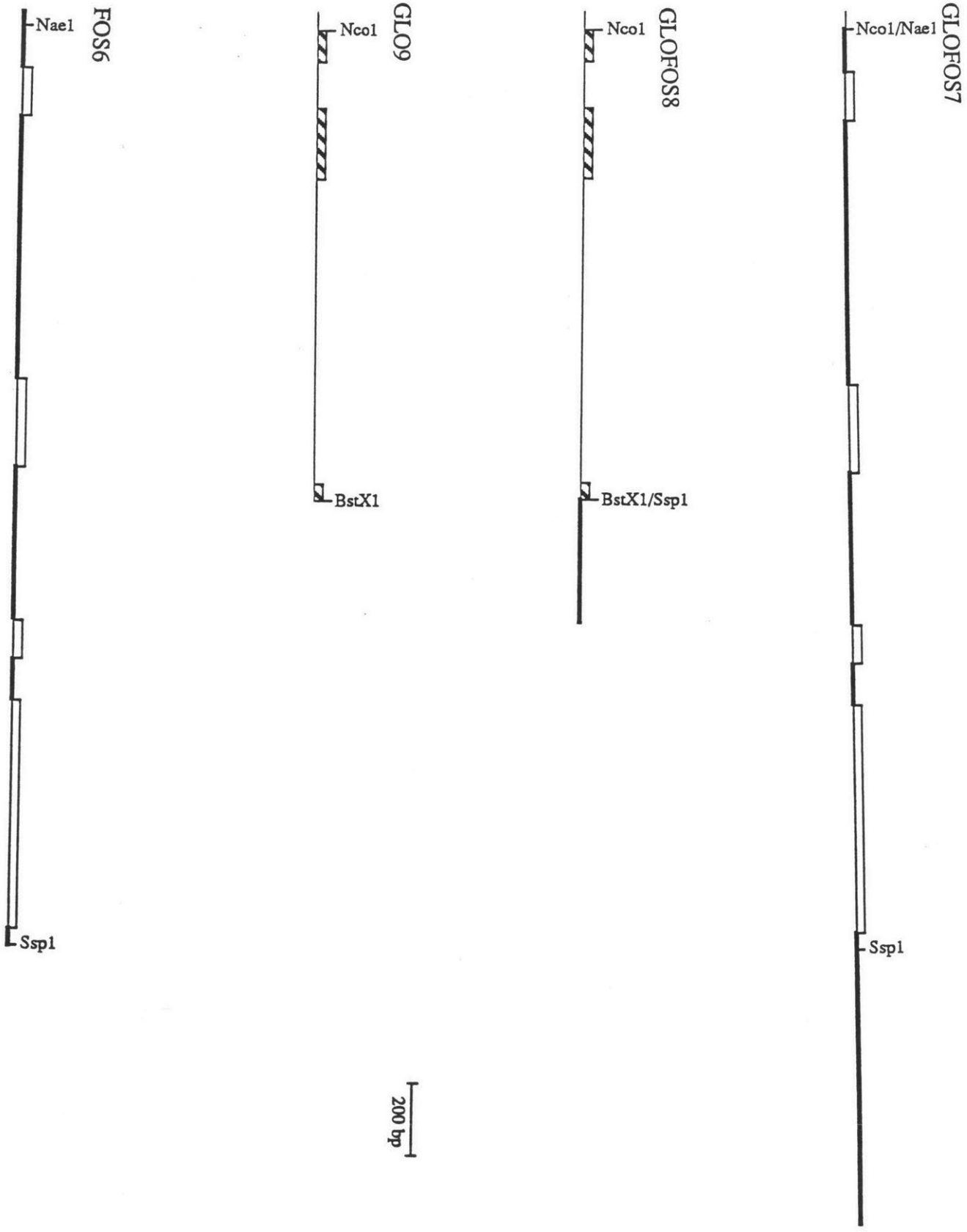

Human c-fos
Nae1
Ssp1
Human ß-globin GLO5
Nco1
BstX1
200 bp
FOSGLO3
Nco1/Nae1
BstX1
FOSGLO4
Nae1
Ssp1/BstX1

Figure FOSN: Genomic, unspliced versions of GLOFOS7, GLOFOS8, GLO9 and FOS6. Thick lines represent fos 5' and 3' untranslated sequences and intervening sequences. Open blocks represent fos protein coding sequences. Thin lines represent globin 5' and 3' untranslated regions and introns. Hatched blocks represent globin protein coding sequences. The scale is shown.


FOS6
Nae1
Ssp1
GLO9
Nco1
BstX1
GLOFOS8
Nco1
BstX1/Ssp1
GLOFOS7
Nco1/Nae1
Ssp1
200 bp

.39 kb Nae1 fragment of human c-fos DNA was used as probe for constructs human c-fos, FOS6, and GLOFOS7. This Nae1-Nae1 fragment was chosen because it diverged from mouse and was high in G-C content. It consists of 255 bases of sequence that appear in the spliced message. It has 14.8% mismatches and a 5 base pair insertion. Figure FOSO illustrates that this probe, when hybridized at 72°C and washed stringently (.1X SSCPE, 60°C) will anneal specifically to human c-fos RNA and not to mouse c-fos RNA. Lanes 1 and 2 contain RNA from JW36 cells, a human attached HeLa cell line, lanes 3 and 4 contain 3T3 cell RNA. All cells were arrested in media containing 5% platelet poor plasma for one day. Lanes 1 and 3 were refed with media containing 15% calf serum. Under the conditions used the only lane that bears signal is the human lane, which had been stimulated to produce fos message. The blot was rehybridized with α-tubulin (Lemischka et al., 1981) to demonstrate that RNA was on the blot in approximately equal quantities in each lane.

Each construct-human c-fos (PCFOS), FOSGLO3, FOSGLO4, FOS6, GLO5, GLOFOS7, GLOFOS8, and GLO9 (Figures FOSA, FOSB, FOSC, FOSD, FOSH, FOSJ, FOSK and FOSL, respectively) was separately cotransfected into Balb/c/3T3 cells along with PSV2NEO (Figure HSPC) by Method B. (See Materials and Methods.) After selection in G418, roughly 24 colonies from each transfection were cloned out. Each cell line was grown up into 3 100mm tissue culture dishes. One dish of each colony was frozen immediately, and the other two were used to screen the cell lines for induction of the fos promoter-driven message. Both of these were fed media containing 5% platelet-poor plasma for one to two days. One of these was then fed media containing 15% calf serum to stimulate the fos promoter. Total cellular RNA was isolated and run on a formaldehyde gel for Northern blot analysis. Figure FOSP shows Northern blots of part of the screens of constructs GLO5

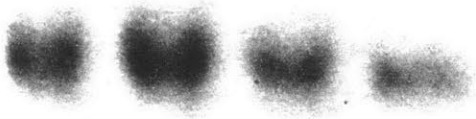
Figure FOSO: Northern blot test to distinguish endogenous mouse c-fos from exogenous human c-fos hybridization. Lanes 1 and 2 contain JW36 RNA, a human attached cell line, lanes 3 and 4 contain 3T3 RNA. All cells were arrested in 5% plasma and only the cells whose RNA is in lanes 1 and 3 were refed media with 15% calf serum. Northern blot was first probed with .39 kb Nae1-Nae1 of human c-fos at 72°C and washed stringently to .1X SSCPE at 60°C (panel A). The blot was rehybridized with rat α-tubulin in the panel B.

A



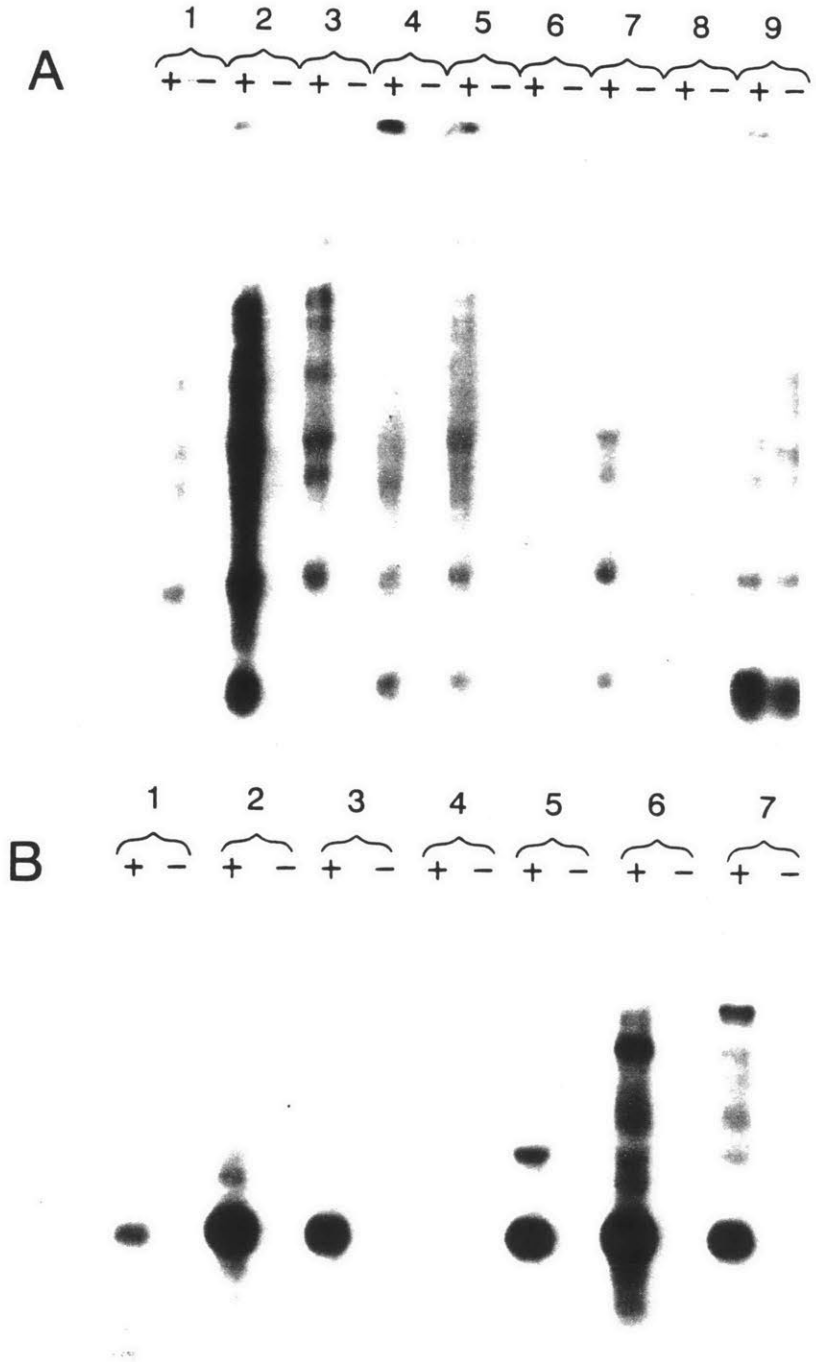
1 2 3 4

B



1 2 3 4

Figure FOSP: Northern blots of part of screens of cell lines harboring constructs GLO5 (A) and GLOFOS8 (B). Each number represents a single cell line. Cells were made quiescent in 5% platelet-poor plasma for 1-2 days and then either not refed (-) or refed media containing 15% calf serum (+) for 30 minutes. Total RNA was extracted and probed with Nco1 to Pst1 fragment of human β-globin.


A
1 2 3 4 5 6 7 8 9
+ − + − + − + − + − + − + − + − + −
B
1 2 3 4 5 6 7
+ − + − + − + − + − + − + −

and GLOFOS8. In most cases there is no transcription detectable in the cells that were not refed media with serum. The uninduced cells usually exhibited no hybridization. In a few cases, signal did exist in lanes with cells that had not been refed (e.g., cell line 9 of A of Figure FOSP). Roughly 20% of the cell lines screened for construct FOSGLO3 and 40% of the cell lines screened for construct GLO5 had a variable degree of signal in the unrefed lanes. No uninduced message is seen in the other constructs which have very short half-lives. As will be seen later, construct GLO5 has the longest lived message in these experiments and construct FOSGLO 3 has the next longest lived cytoplasmic RNA. The relatively high level of message detected in an uninduced state of the cell, which correlates with the half-life of the message, could be explained by the half-life itself. If a given amount of constitutive expression is promoted by the fos promoter, the longer-lived the message the more of it will be detectable at any given moment. Higher molecular weight species of RNA were observed in the total cellular RNA used in screening the cell lines, as was noted earlier in Chapter II on heat shock. These species disappeared when cytoplasmic RNA was used. In addition, some transcripts of aberrant size were detected in some cases.

A scaled, comparative representation of the expected messages driven from the fos promoter are given in Figures FOSQ and FOSR. Beside each message is the average half-life in minutes as presented in Tables FOSA through FOSI. Chimeras between fos and globin were used to map those sequences which determine RNA half-lives. The breakpoint sites were chosen so as to avoid disruption of the fos and globin proteins as best as possible. In no case was splicing interrupted. In all cases but one, GLO9, the messages were the expected sizes based on comparison to 28S and 18S ribosomal bands on the photographs of the gels prior to transfer. All

Figure FOSQ: Schematic RNA structures accompanied by their average half-lives of human c-fos, GLO5, FOSGLO3 and FOSGLO4. Thick lines represent 5' and 3' untranslated regions of fos. Open blocks represent fos protein coding sequences, exon boundaries marked. Thin lines represent 5' and 3' untranslated regions of globin. Hatched blocks represent globin protein coding sequences, exon boundaries marked. The scale is shown. Half-lives are given in minutes beside each structure.

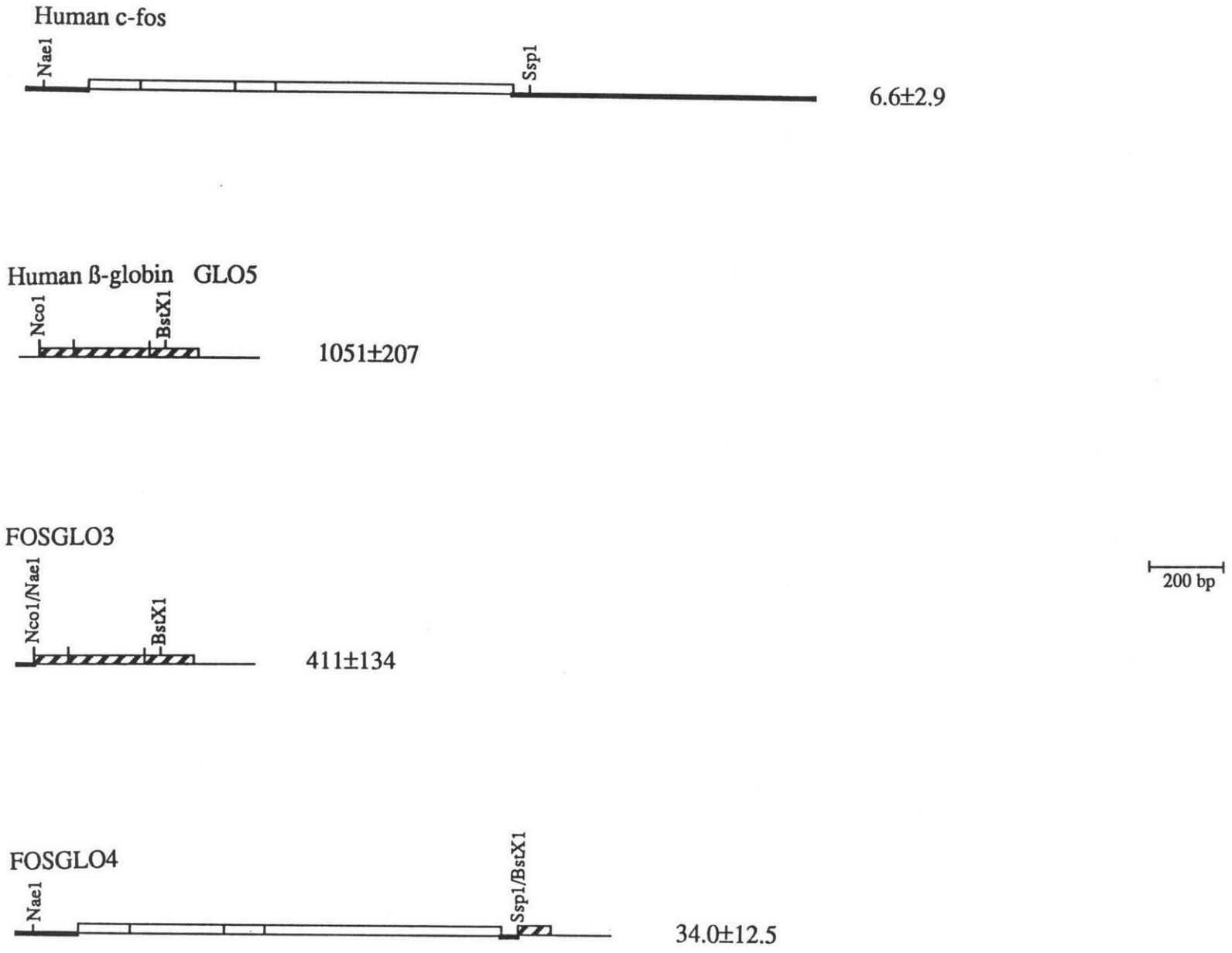

Human c-fos
Nae1
Ssp1
6.6±2.9
Human ß-globin GLO5
Nco1
BstX1
1051±207
FOSGLO3
Nco1/Nae1
BstX1
411±134
200 bp
FOSGLO4
Nae1
Ssp1/BstX1
34.0±12.5

Figure FOSR: Schematic RNA structures accompanied by their average half-lives of GLOFOS7, GLOFOS8, GLO9 and FOS6. Thick lines represent 5' and 3' untranslated regions of fos. Open blocks represent fos protein coding sequences, exon boundaries marked. Thin lines represent 5' and 3' untranslated regions of globin. Hatched blocks represent globin protein coding sequences, exon boundaries marked. The scale is shown. Half-lives are given in minutes beside each structure.

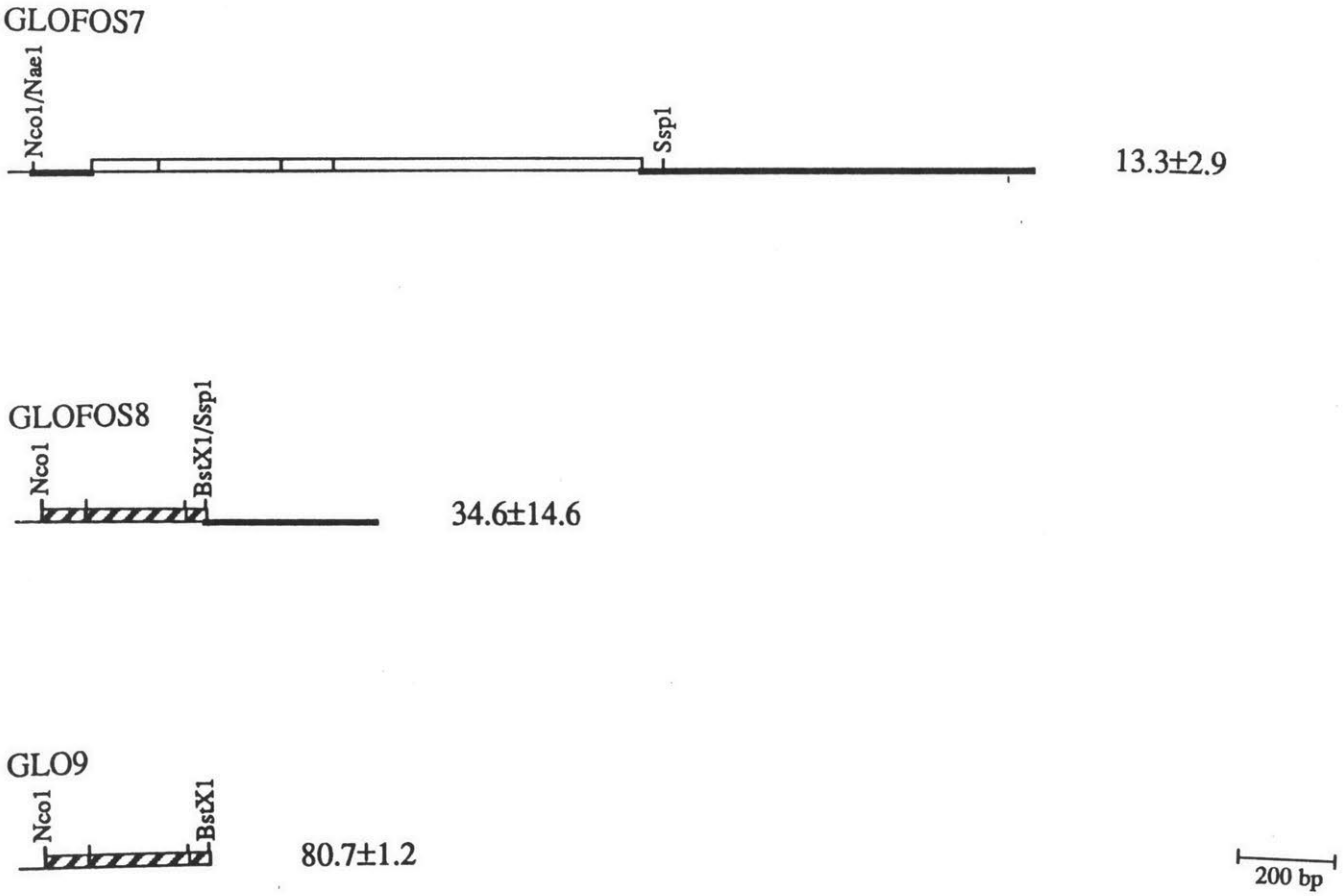

GLOFOS7
Nco1/Nae1
Ssp1
13.3±2.9
GLOFOS8
Nco1
BstX1/Ssp1
34.6±14.6
GLO9
Nco1
BstX1
80.7±1.2
200 bp
FOS6
Nae1
Ssp1
42.6±6.5

Table FOSA

HUMAN C-FOS

CELL LINE	EXPT.	BLOT	1/2-LIFE±S.D.[5]	t[1]	D.F.[2]	P[3]	%CONFLUENT	AVERAGE±S.D.[4,5]
1C2[6]	4/7/86	5/19/86Aa	-	-	-	-	100	
1C2[7]	9/28/86	10/25/86A	14.1±13.2	1.1	4	.3<P<.4	100	
1C2	9/28/86	4/29/87B	9.0±0.8	11.4	8	<.001	100	
1C6	1/18/87	2/2/87B	3.3±0.1	47.3	2	<.001	100	
1C6	3/31/87	4/15/87A	7.5±0.2	39.6	4	<.001	100	6.6±2.9

1 t-statistic (See Appendix)
2 degrees of freedom (See Appendix)
3 P-value ≤ .05 is acceptable (SeeAppendix)
4 average of statistically acceptable half-lives
5 half-lives in minutes
6 only two points on line of decay
7 probably due to artifact of transfer as 4/29/87B blot of same RNA gave acceptable P-value

Table FOSB

GLO5

CELL LINE	EXPT.	BLOT	1/2-LIFE±S.D.[5]	t[1]	D.F.[2]	P[3]	%CONFLUENT	AVERAGE±S.D.[4,5]
B5b[6]	9/2/86	9/19/86B	-	-	-	-	100	
C5b[7]	12/7/86	12/12/86D	302±264	1.1	2	.2<P<.4	100	
B3a[6]	1/14/87	1/27/87A	-	-	-	-	100	
2d1[6]	2/3/87b	2/12/87B	-	-	-	-	30	
2d1	2/3/87c	2/12/87B	914±145	6.3	8	<.001	100	
2d1	1/31/87	2/13/87D	950±150	6.3	4	.001<P<.005	85	
2d1	4/28/87	4/30/87	1289±364	3.5	6	.01<P<.025	100	1051±207

1 t-statistic (See Appendix)
2 degrees of freedom (See Appendix)
3 P-value ≤ .05 is acceptable (SeeAppendix)
4 average of statistically acceptable half-lives
5 half-lives in minutes
6 only 1-2-fold induction of message levels
7 not statistically acceptable, only about 5-fold induction of message, only 3 points on line of decay, scattered

Table FOSC

FOSGLO3

CELL LINE	EXPT.	BLOT	1/2-LIFE±S.D.[5]	t[1]	D.F.[2]	P[3]	%CONFLUENT	AVERAGE±S.D.[4,5]
3c4b	3/3/86	3/18/86	583±94	6.2	2	.025	85	
3c4b	4/1/86	5/19/86	258±48	5.4	5	.001<P<.005	95	
3b1a	1/14/87	2/2/87A	384±65	5.9	4	.001<P<.005	30	
3b1a	4/28/87	4/30/87	420±39	10.8	4	<.001	100	411±134

1 t-statistic (See Appendix)
2 degrees of freedom (See Appendix)
3 P-value ≤. 05 is acceptable (SeeAppendix)
4 average of statistically acceptable half-lives
5 half-lives in minutes

Table FOSD

FOSGLO4

CELL LINE	EXPT.	BLOT	1/2-LIFE±S.D.[5]	t[1]	D.F.[2]	P[3]	%CONFLUENT	AVERAGE±S.D.[4,5]
5a1[6]	6/10/86	6/16/86A	-	-	-	-	100	
5a1	7/15/86	8/15/86A	27.4±2.5	11.0	6	<.001	100	
5a1	1/13/87	3/4/87	26.1±1.8	14.8	6	<.001	100	
5d3[7]	3/12/87	4/2/87A	49.3±8.1	6.1	6	<.001	100	
5d3[7]	3/12/87	4/2/87B	48.4±0	∞	7	<.001	100	34.0±12.5

1 t-statistic (See Appendix)
2 degrees of freedom (See Appendix)
3 P-value ≤ .05 is acceptable (SeeAppendix)
4 average of statistically acceptable half-lives
5 half-lives in minutes
6 only two data points on line of decay
7 only one version of this experiment was used in calculating the average

Table FOSE

GLOFOS7

CELL LINE	EXPT.	BLOT	1/2-LIFE±S.D.[5]	t[1]	D.F.[2]	P[3]	%CONFLUENT	AVERAGE±S.D.[4,5]
A3a	2/5/87	2/13/87C	12.0±3.8	3.1	2	.05<P<.1	100	
A3a	2/26/87a	3/3/87C	11.3±2.2	5.2	7	.001<P<.005	30	
A3a	2/26/87b	3/3/87D	16.7±3.7	4.5	5	.005<P<.01	100	13.3±2.9

1 t-statistic (See Appendix)
2 degrees of freedom (See Appendix)
3 P-value ≤ .05 is acceptable (SeeAppendix)
4 average of statistically acceptable half-lives
5 half-lives in minutes

Table FOSF

GLOFOS8

CELL LINE	EXPT.	BLOT	1/2-LIFE±S.D.[5]	t[1]	D.F.[2]	P[3]	%CONFLUENT	AVERAGE±S.D.[4,5]
C3b	11/19/86	11/26/86A	52.8±8.8	6.0	8	<.001	100	
C3b-a	2/8/87a	2/12/87A	39.9±11.0	3.6	8	.005<P<.01	30	
C3b-b	2/8/87b	2/12/87A	23.2±1.8	13.0	4	<.001	100	
A2c	3/27/87	4/3/87	22.3±4.8	4.7	5	.005<P<.01	100	34.6±14.6

1 t-statistic (See Appendix)
2 degrees of freedom (See Appendix)
3 P-value ≤ .05 is acceptable (SeeAppendix)
4 average of statistically acceptable half-lives
5 half-lives in minutes

Table FOSG

GLO9

CELL LINE	EXPT.	BLOT	1/2-LIFE±S.D.[5]	t[1]	D.F.[2]	P[3]	%CONFLUENT	AVERAGE±S.D.[4,5]
B1b	11/23/86	11/26/86B	81.7±5.5	14.8	6	<.001	100	
B1b-a	2/18/87a	3/3/87A	79.4±15.5	5.1	5	.001<P<.005	30	
B1b-b	2/18/87b	3/3/87B	80.9±12.6	6.4	8	<.001	100	80.7±1.2

1 t-statistic (See Appendix)
2 degrees of freedom (See Appendix)
3 P-value ≤ .05 is acceptable (SeeAppendix)
4 average of statistically acceptable half-lives
5 half-lives in minutes

Table FOSH

FOS6

CELL LINE	EXPT.	BLOT	1/2-LIFE±S.D.[5]	t[1]	D.F.[2]	P[3]	%CONFLUENT	AVERAGE±S.D.[4,5]
II3b	6/14/86	6/16/86B	47.2±4.5	10.5	5	<.001	100	
II3b	7/18/86	8/15/86B	38.0± 4.8	8.0	5	<.001	100	42.6±6.5

1 t-statistic (See Appendix)
2 degrees of freedom (See Appendix)
3 P-value ≤ .05 is acceptable (SeeAppendix)
4 average of statistically acceptable half-lives
5 half-lives in minutes

Table FOSI

MOUSE C-FOS

CELL LINE	EXPT.	BLOT	1/2-LIFE±S.D.[5]	t[1]	D.F.[2]	P[3]	%CONFLUENT	AVERAGE±S.D.[4,5]
8-C3b	11/19/86	11/26/86A	12.0±.5	7.8	2	.01<P<.025	100	
3T3	11/24/85	12/5/85	15.8±.8	19.1	3	<.001	100	
7-A3a	2/26/87b	4/29/87C	13.1±2.8	4.6	5	.005<P<.01	100	
hu c-fos1C6	3/31/87	4/15/87B	7.7±.6	13.0	4	<.001	100	
hu c-fos1C2	9/28/86	4/29/87A	21.5±3.1	7.0	8	<.001	100	14.0±5.1

1 t-statistic (See Appendix)
2 degrees of freedom (See Appendix)
3 P-value ≤ .05 is acceptable (SeeAppendix)
4 average of statistically acceptable half-lives
5 half-lives in minutes

constructs, except GLO9 and FOS6, used the poly(A) addition signal carried on the 3' end of that message. For human c-fos, GLOFOS8 and GLOFOS7, the poly(A) signal from the human c-fos gene was used. For GLO5, FOSGLO3 and FOSGLO4, the poly(A) signal from the human β-globin gene was used. GLO9 and FOS6 were constructed to determine the effects of the loss of 3' sequences. The poly(A) signals on each of these constructs were also removed. Since there was no simple way of deleting the 3' region while keeping the poly(A) signal intact (replacing it with a SV40 poly(A) signal cassette would change the inherent structure of the message), the constructs were left with no poly (A) signal.

Determination of RNA Half-Lives:

Kinetic experiments to assess the stability of a fos promoter-driven mRNA (fos, globin, or chimeras between the two) were performed by refeeding different plates of a given cell line for increasing amounts of time, making cytoplasmic RNA, and quantifying the specific signal detected on Northern blots made from these RNAs (as described in detail in the Appendix). A preliminary time course was first chosen based on the expected half-life of the message. For a second experiment of a given construct the time course was often adjusted to observe as many points as possible on the line of decay. In most cases the maximum amount of hybridization occurred at 30 to 60 minutes after induction. In a few cases, for constructs GLO5 and FOSGLO3, the maximum intensity of hybridization occurred at 2 to 4 hours after induction. This appears to be related to the longer half-lives of these messages. A cell line was chosen from the screen of the construct for high induction of the fos promoter-driven message and for having a major transcript at the expected size. The cell line was thawed and grown up, in most cases, to 10 150mm tissue

culture dishes. All 10 plates were given media containing 5% platelet-poor plasma for 1 to 3 days. Nine of the ten plates were refed media containing 15% calf serum at staggered intervals. Four examples of such experiments are supplied in Figures FOSS through FOSV. Panel A of each figure is a Northern blot of the time course of RNAs probed with the DNA which hybridized specifically to the fos-induced message. Panel B is the same Northern blot reprobed with the normalizing rat α-tubulin cDNA. Panel C is the best-fit line of the log of the absorbances of the bands normalized to tubulin and plotted against time as described in the Appendix. The highest intensity normalized band is taken as the starting point, and the lowest intensity normalized band above constitutive level is taken as the final time point. Only in constructs GLO5 and FOSGLO3 was there any detectable constitutive expression. Details are described in the Figure Legends.

The data for each construct are summarized in Tables FOSA through FOSI. In the few cases that more than one experiment of the same cell line was performed on the same day, each experiment was supplied with a different letter following the date. The half-life of the endogenous mouse c-fos transcript was determined by reprobing blots of other experiments for which a useful time course had been used with mouse c-fos. Only experiments from which adequate amounts of RNA were obtained are discussed. Of 31 sets of RNAs, the 26 which gave acceptable P-values as described in the Appendix, are used in calculating the averages and standard deviations. In three cases, there was no induction of the message. Three different cell lines of GLO5, all of which were inducible upon screening, did not induce well enough during the experiment. As will be discussed later, one of these probably did not induce because the cells were sparse. In two cases, an experiment was discarded because only two data points were available on the line of decay, as the wrong time course was chosen for the

Figure FOSS: Experiment FOSGLO3, 3b1a, blot 2/2/87A. (See Table FOSGLO3.) A Northern blot of cytoplasmic RNAs extracted from these cells after 0, 120, 420, 510, 600, 720, 1230, and 1590 minutes (lanes 1-8, respectively) of refeeding with media containing 15% calf serum after 2 days of growth arrest in 5% plasma. Panel A represents the blot hybridized with the 2.6 kb Nco1 to Pst1 segment of HUBGLO (Figure FOSB). Panel B represents that same blot reprobed with rat α-tubulin. Panel C shows the best-fit line of the normalized globin absorbances plotted against time.

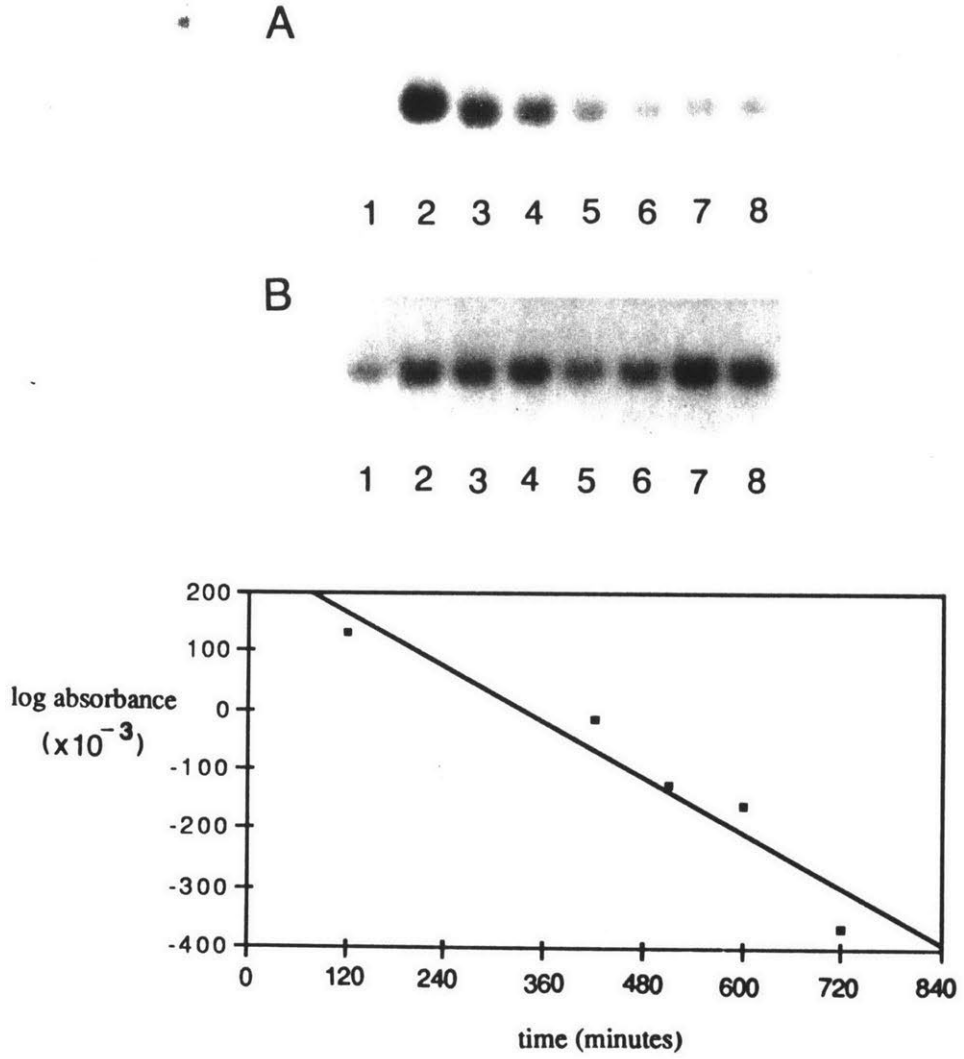
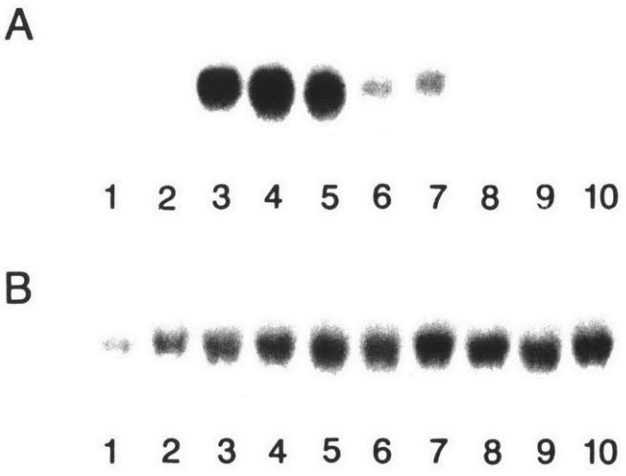

A
1 2 3 4 5 6 7 8
B
1 2 3 4 5 6 7 8
log absorbance
(x10⁻³)
200
100
0
-100
-200
-300
-400
0
120
240
360
480
600
720
840
time (minutes)

Figure FOST: Experiment FOSGLO4, 5a1, blot 8/15/86A. (See Table FOSGLO4.) A Northern blot of cytoplasmic RNAs extracted from these cells after 0, 15, 30, 45, 60, 75, 90, 105, 120, and 150 minutes (lanes 1-10, respectively) of refeeding with media containing 15% calf serum after 2 days of growth arrest in 5% plasma. Panel A represents the blot hybridized with the 2.6 kb Nco1 to Pst1 segment of HUBGLO (Figure FOSB). Panel B shows the the same blot reprobed with rat α-tubulin. Panel C shows the best-fit line of the normalized globin absorbances plotted against time.


A
1 2 3 4 5 6 7 8 9 10
B
1 2 3 4 5 6 7 8 9 10

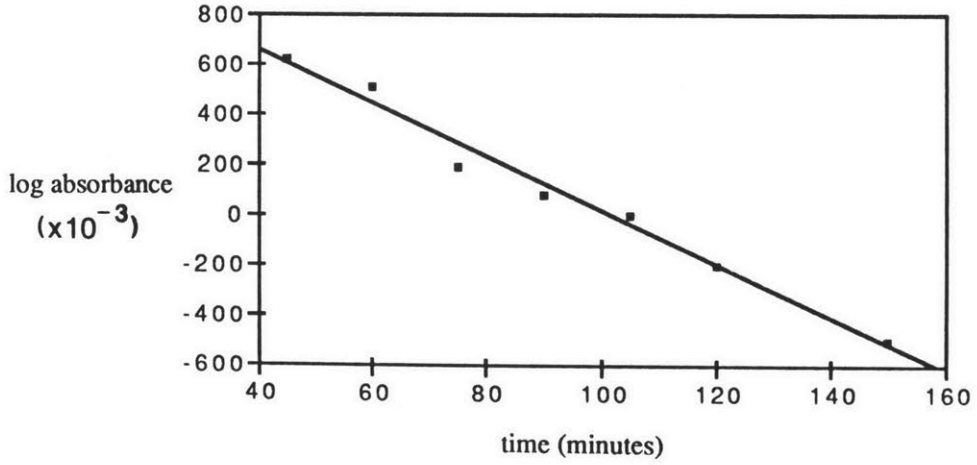

800
600
400
200
0
-200
-400
-600
log absorbance
(x10⁻³)
40
60
80
100
120
140
160
time (minutes)

Figure FOSU: Experiment of human c-fos, 1C6, blot 4/15/87A. (See Table HUMAN C-FOS.) A Northern blot of cytoplasmic RNAs extracted from these cells after 0, 25, 30, 35, 40, 45, 50, 55, and 60 minutes (lanes 1-9, respectively) of refeeding with media containing 15% calf serum after 2 days of growth arrest in 5% plasma. Panel A represents the blot hybridized with the .39 kb Nae1 fragment of human c-fos (see legend to Figure FOSO). Panel B shows the same blot rehybridized with rat α-tubulin. Panel C shows the best-fit line of the normalized fos absorbances plotted against time.

A



1 2 3 4 5 6 7 8 9

B



1 2 3 4 5 6 7 8 9

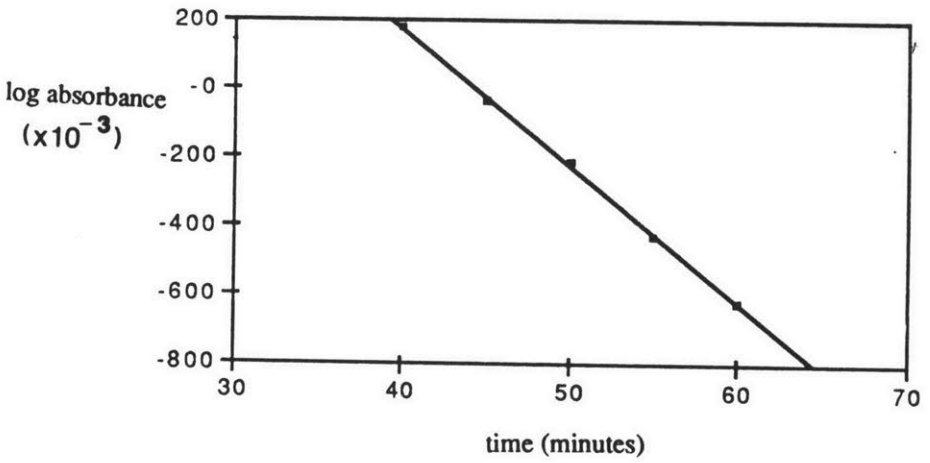


Figure FOSV: Experiment of human c-fos, 1C6, blot 4/15/87B, probed with mouse c-fos. (See Table MOUSE C-FOS.) A Northern blot of cytoplasmic RNAs extracted from these cells after 0, 25, 30, 35, 40, 45, 50, 55, and 60 minutes (lanes 1-9, respectively) of refeeding with media containing 15% calf serum after 2 days of growth arrest in 5% plasma. Panel A represents the blot hybridized with mouse c-fos DNA under normal conditions. Panel B shows the same blot rehybridized with rat α-tubulin. Panel C shows the best-fit line of the normalized mouse c-fos absorbances plotted against time.

A

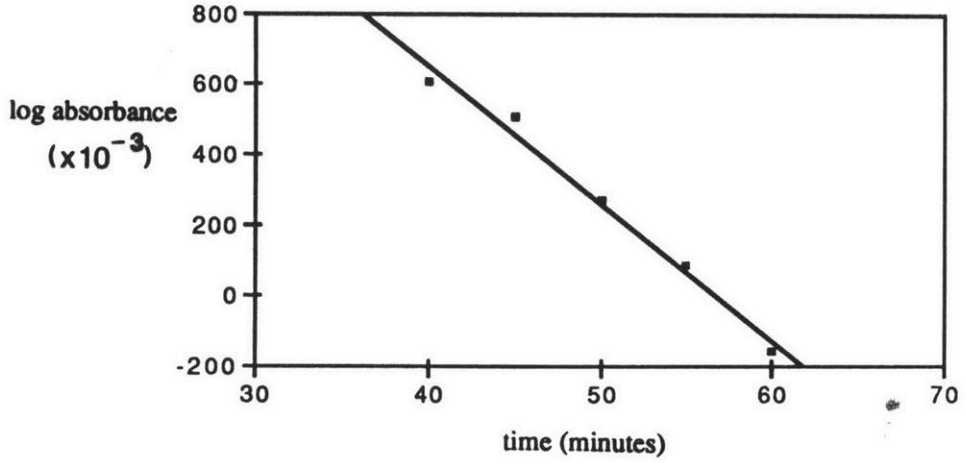


1 2 3 4 5 6 7 8 9

B



1 2 3 4 5 6 7 8 9



first experiment. In only 1 experiment, GLO5 C5b, were the data too scattered to use.

Several points are worthy of note. The blots are reproducible, as is illustrated in Table FOSD of FOSGLO4. The 3/12/87 experiment of cell line 5d3 was run on two blots which gave identical half-lives. Only one version was incorporated into the average half-life. Different cell lines harboring the same construct gave reproducible results. The two cell lines used for experiments on FOSGLO3, 3b1a and 3c4b, gave comparable half-lives, as did the cell lines used for construct GLOFOS8. Any lack of reproducibility did not correspond with the cell line, but with the particular experiment.

The summarized results are given in Figures FOSQ and FOSR. The overall results extracted from these data are that sequences which signal the cell to degrade human c-fos and/or protect human β-globin in 3T3 cells appear to be located along the entire length of the structural message. This will be further discussed later. GLO9 and FOS6 are shown because, despite their lack of a poly(A) signal, they each have been observed as a single molecular weight species in the cytoplasm. It is possible that they have picked up a signal from the plasmid or in contradiction to conventional wisdom, have become cytoplasmic entities without a poly(A) tail. As explained above, these constructs were intended to control for the effect of deleting a given piece of DNA. Another caveat for interpretation of these results is that the primary sequences of the DNAs being exchanged are only part of what must be considered. Disruption of the junctions, as well as removal of the DNA from its natural host can also compound the effect on message stability of transposition of the DNA. The secondary and tertiary structure in the context of the entire message may also be of issue.

In order to assess the effects of the 3' end of these messages on their half-lives the following comparisons must be made. In comparing human c-fos and FOSGLO4, it can be seen that replacing the 3' untranslated region of fos with the 3' untranslated region of globin plus a small stretch at the end of its third exon, had a four-fold stabilizing effect on the basic fos message. In the reciprocal chimera, in which the 3' end of fos replaced the 3' end of globin, comparing GLO5 (human β-globin) and GLOFOS8, the effect is even more dramatic. The 3' untranslated region of fos had a 30-fold destabilizing effect on the basic globin message.

To consider the effects on message stability afforded by the 5' untranslated regions of fos and globin, comparison of (1) GLO5 and FOSGLO3 and (2) human c-fos and GLOFOS7 must be made. In the former case, the entire globin 5' untranslated region was replaced by the first 40 bases of the fos message, including its cap. This replacement destabilized globin message 2.5-fold. In the latter case, there is no apparent effect of replacing those 40 bases of fos with the entire 50 base 5' untranslated region of globin. Since the half-lives of these messages are so short, it is difficult to say whether they are statistically different.

To assess the input of the body of the message on inherent stability two comparisons between constructs can be made. In comparing FOSGLO3 and FOSGLO4, the coding regions of the hybrids are transposed. There is a 12-fold destabilizing effect conferred by the coding sequences of fos. (It can also be considered that the coding region of globin confers a 12-fold stabilizing effect.) In comparing GLOFOS7 and GLOFOS8, the same transposition has been made. In this case, there is only a 2.5-fold destabilizing effect conferred by the fos coding sequences. This result strongly suggests that it is the context of the sequence and/or the actual junctions that were destroyed which is influential in determining RNA stability.

State of Confluence:

As discussed in the Chapter IB1 there was reason to believe that the state of confluence of the cells could affect the half-lives of the messages. Most experiments were performed on confluent cells as indicated in Tables FOSA through FOSI. Experiments in the literature (Greenberg and Ziff, 1984; Muller et al., 1984) that showed the induction of fos message and other competence genes in response to refeeding after growth arrest had been performed on confluent cells. Five constructs were considered with respect to cells at various states of confluence (GLO5, GLO9, FOSGLO3, GLOFOS7, and GLOFOS8, see Tables FOSB, FOSG, FOSC, FOSE, and FOSF). The results were included together with the data on confluent cells because there was no difference in half-lives. In the case of GLO5, cell line 2d1 did not induce the fos-driven message in 30% confluent cells on the same day that confluent cells, which had been grown up in parallel, induced well. While sparse cells might have been prevented from inducing the fos-driven message in this case, in other cases the fos promoter did induce quite well in a sub-confluent state.

Growth of the Cells:

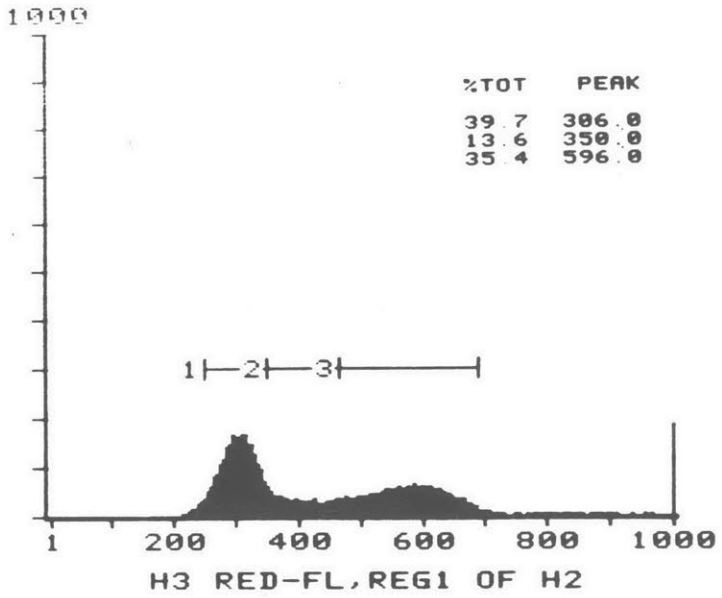
Several additional control experiments were performed. As discussed in the Chapter IB1, the rate of growth of a cell may affect the overall rate of message degradation. Differential growth from cell line to cell line could thus cause variability in stability. In addition, any growth of the cells during the course of the experiment would dilute the specific message being addressed, especially in longer experiments. Equal RNA was loaded on each lane of a Northern gel, so the same amount of RNA from more cells would consist of less of the induced message if the cells had grown. The precise growth rate must be known in order to adjust the concentration

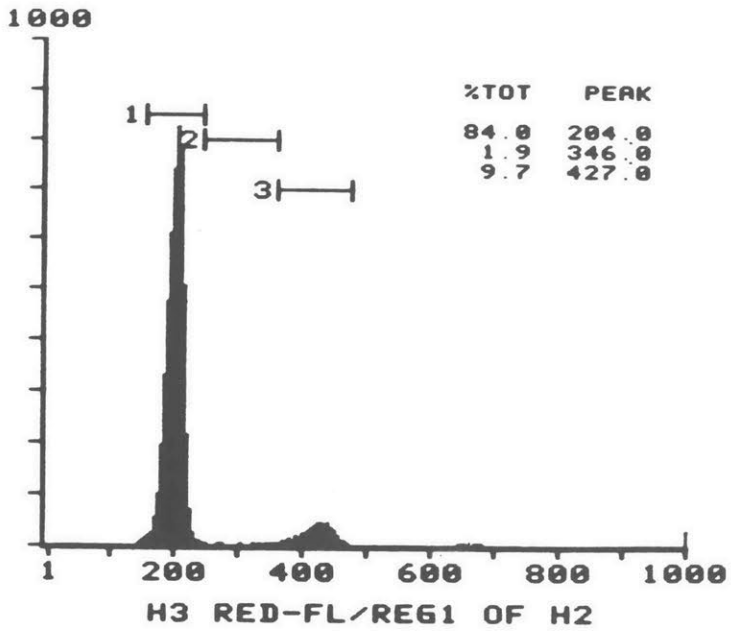
of RNA. Since different experiments did not use the same time course for obtaining a half-life it was impossible to run a mouse c-fos endogenous control on every experiment to adjust for this. Although the cells were untransformed and therefore, growth arrested at confluence, prior to transfection, it was possible that after transfection, selection, freezing, and thawing, they could have become slightly transformed and actually have grown somewhat in response to refeeding with calf serum.

In order to address the question of whether the cells were undergoing mitosis, several cell lines harboring different constructs were thawed, grown and each plated into 15 60 mm plates. An experimental protocol was followed in which the cells were grown to confluence, treated with 5% plasma for two days and then refed with 15% calf serum for up to 54 hours. At random intervals during those treatments, a plate of cells was removed from the incubater, trypsinized and fixed in ethanol. When all the plates were fixed, the samples were stained with propidium iodide which binds nucleic acid. RNA was destroyed with RNase. The samples were then run through an Ortho Cytofluorograph 50H (Ortho Diagnostic Systems, Westwood, Mass.) by V. Falco in the Cell Sorter Laboratory, MIT, in order to obtain a quantitative evaluation of the DNA content of the cells.

These data were then interpreted quantitatively to determine the percentage of cells in S-phase and hence, the growth rate of the cells. Figure FOSW shows two histograms of the output from the cytofluorograph. The x-axis represents the amount of red fluorescence, which correlates exactly with the DNA content of a given cell. The y-axis represents the number of cells with that DNA content. The left-hand peak represents cells in G_0/G_1 of the cell cycle, the trough between the peaks represents cells in S phase, and the right-hand peak represents cells in G_2/M. The percentage of cells in each region is marked within each histogram next to

Figure FOSW: Cytofluorimetry experiment. Cell from various points of growth, arrest in plasma, and refeeding in calf serum were trypsinized, fixed, RNsed and stained in 50 μg/ml propidium iodide as described in Materials and Methods. The samples were processed for amount of fluorescence in an Ortho Cytofluorimeter in the Cell Sorter Laboratory by V. Falco. The x-axis of the histogram represents the amount of DNA based on fluorescence of given cell. The y-axis represents the number of cells with that DNA content. The upper histogram is representative of a population of growing cells. The lower histogram is representative of a population of stationary cells.


1000
%TOT PEAK
39.7 306.0
13.6 350.0
35.4 596.0
1
2
3
1
200
400
600
800
1000
H3 RED-FL,REG1 OF H2


1000
%TOT PEAK
84.0 204.0
1.9 346.0
9.7 427.0
1
2
3
1
200
400
600
800
1000
H3 RED-FL/REG1 OF H2

the fluorescence value of that peak. The average fluorescence in the right-hand peak is roughly twice that of the left-hand peak, as would be expected based on DNA content. The upper panel shows an exemplary histogram of growing cells in which 39.7% of the cells were in G_0/G_1, 13.6% of the cells were in S phase, and 35.4% were in G_2/M. The lower histogram is characteristic of all the analyses performed on confluent, transfected, selected cell lines after various times of refeeding (up to 54 hours) following a two-day growth arrest in plasma. 84% of the cells were in G_0/G_1, 1.9% of the cells were in S, and 9.7% of the cells were in G_2/M. This pattern of fluorescence is typical of 3T3 cells that are not growing, as there are negligible numbers of cells in S and relatively few cells in G_2/M compared with growing cells. These results indicate that any variability from experiment to experiment was not due to growth and that dilution of the message being considered should not be an issue of concern.

Translation:

Since the translation of an RNA may affect its stability (as discussed in Chapter IB2) the chimeric messages were tested for translation. The approach chosen to assess whether these RNAs were active messengers was to probe fractions of polysome gradients of cells induced to produce the fos-driven RNA with DNAs specific to the chimeric message. Cytoplasmic extracts of four different cell lines, harboring 4 different constructs (human c-fos, FOSGLO3, FOS6 and GLOFOS7) were run through 10-50% sucrose gradients. Fractions were collected and pooled such that RNPs and the 40S ribosomal subunit were in the RNP fraction, the 60S ribosomal subunit accompanied the monosomes and disomes, and polysomes were pooled together. The optical density profile along with the rate of scanning permitted accurate poolings based on these criteria. GLOFOS7 was refed for 35

minutes, FOS6 and human c-fos were refed for 40 minutes, and FOSGLO3 was refed for two hours since, as described earlier, the maximum intensities of hybridization occur at these times. The four constructs include a message that has no apparent poly(A) addition signal (FOS6), a message that uses the "perfect" globin capping site at its 5' end (GLOFOS7) as well as two messages that use the fos capping site. As will be discussed below, a significant amount of polysomal material was obtained only from cells harboring construct FOSGLO3. Figure FOSX has three equally loaded Northern blots containing mRNPs (lane 1) and monosomes and disomes (lane 2) of 35 minute refed GLOFOS7 containing cells, A3a. The blot in Panel A was hybridized with .39 kb human c-fos probe to determine the behavior of the fos-driven chimeric message. The blot in Panel B was hybridized with a mouse c-fos probe to assess the behavior of the endogenous transcript. It is evident that both messages are not in the RNP fraction and that upon synthesis and extrusion into the cytoplasm they are immediately sequestered onto translational machinery. Clearly, the chimeric message behaved in a similar manner to the endogenous transcript. All other exogenous messages tested exhibited identical behavior. Therefore, since chimeric RNAs are on polysomes all RNAs analyzed herein are "mRNA"s.

Negligible polysomal material was isolated from cells that had been induced for a short time (cells harboring constructs human c-fos, FOS6, and GLOFOS7). A significant amount was purified from cells that had been refed media containing 15% calf serum for two hours (FOSGLO3). The polysomal optical density profiles mirrored these results, suggesting that during cellular growth arrest, as during amino acid deprivation, polysomes disassociate, leaving RNAs in RNPs (Baliga et al., 1969). One could argue that the polysomes are in the process of being reaggregated as the time of refeeding increases. The blot in Panel C of Figure FOSX was hybridized

Figure FOSX: Northern blots of RNA from polysome gradients. Fractions were collected from 10-50% sucrose gradients containing cytoplasmic extract from cells harboring construct GLOFOS7, A3a, after 35 minutes of refeeding with 15% calf serum following two days of growth arrest in plasma. Fractions were pooled into 3 sections: mRNPs (lane 1), monosomes and disomes (lane 2), and polysomes (lane 3). Very little polysomal material was obtained, probably because there was very little after 35 minutes of calf-serum refeeding. All three panels were loaded equally with 8 μg of RNA per lane. The blot in Panel A was hybridized with the human c-fos specific probe (.39 kb Nae1 fragment of human c-fos). The blot in Panel B was hybridized with the mouse c-fos probe. The blot in Panel C was hybridized with rat α-tubulin.

A



1 2

B



1 2

C



1 2

with rat α-tubulin, a message that is present in equal quantities throughout growth arrest and refeeding (Thomas and Thomas, 1986). It was therefore useful as a normalizing control. The presence of approximately equal hybridization in lanes one and two suggests that the message is in the process of returning to polysomes from its stored state on RNPs during growth arrest.

Discussion

Half-Lives of Chimeric RNAs:

In these studies, the human c-fos promoter induced homologous DNA to transcribe homologous message, as well as various heterologous chimeric messages of fos and globin, including those that employed the globin capping sequence in the mouse cell. The kinetics and magnitude of this heterologous stimulation were comparable to the endogenous c-fos response. RNA half-lives were obtained using this system and corroborate data on identical messages in the same cells under the same conditions. Rahmsdorf et al. (1987) state that the half-life of mouse c-fos mRNA after stimulation of serum starved cells is 9 minutes while the results obtained herein are 14.0 +/- 5.1 minutes. As indicated in Chapter IE, there are no definitive data on the half-life of β-globin message in fibroblasts. Its half-life in MEL cells, induced to differentiate, is roughly 60 hours, as compared with our estimate of 17.5 hours in fibroblasts. This suggests that messages have characteristic half-lives for particular cells under particular conditions. In addition, half-lives obtained from the set of fos-globin chimeras revealed that sequences important for the control of RNA stability are located throughout the lengths of these messages.

The kinetics of heterologous fos stimulation between constructs are comparable but, not identical. GLO5 and FOSGLO3 reached their maximal levels at about two hours post-stimulation, probably due to their longer half-lives. The shorter-lived messages all reached their maximum levels within one hour in the same time span as endogenous mouse c-fos and exogenous human c-fos. The magnitude of the stimulation was not quantified, but in all cases there was enough message to be easily measured. The induction over basal levels was also comparable, except in the cases of the long-lived messages, GLO5 and

FOSGLO3. The high constitutive level of expression made determination of the half-life of GLO5 difficult. Since the message is longer-lived, a larger given amount of it might exist in the cytoplasm at a given moment, assuming that the tightness of control over the promoter is constant from construct to construct. In a situation where a large quantity of a stable message is required after stimulation and not at all under unstimulated conditions, there would have to be stricter transcriptional control over the promoter than there is over the fos promoter.

Shaw and Kamen (1986) have found an AU-rich 51 base pair sequence located in the 3' untranslated regions of various lymphokine, cytokine and proto-oncogene RNAs which they claim are the recognition sequences that signal an mRNA processing pathway to specifically degrade them. The largest common motif, AUUUA, appears from 1 to eight times in the 3' untranslated region of many of these RNAs. It occurs only 1 time in human c-myc and human c-myb RNAs and only 2 times in four others (human α-interferon, human β-interferon, human lymphotoxin and IgG1 induction factor). These authors failed to mention that this motif also is present in human β-globin, rabbit β-globin, mouse β-minor globin and mouse β-major globin RNAs, all relatively stable messages. In light of the presence of this motif in stable messages, it is clear that there must be additional signals/sequences involved to account for the instability of the fos message. The significance of this region was indicated by Meijlink et al., (1985), when they deleted a 67 base pair region of the 3' untranslated region of fos, which included the Shaw/Kamen sequence, and converted the proto-oncongene to a transforming gene. Kamen and Shaw provided no quantitative evaluation of the effect of transposing a region of DNA. Though these authors used a 51 nucleotide region which contained their 5-mer, they attribute their results to the 5-mer sequence. The data herein provide quantitative evidence that the sequences in the 3' untranslated region of

human c-fos, are not the only sequences involved in determining message stability.

When most of the 3' untranslated region of fos replaces the 3' untranslated region of globin, (GLO5 vs. GLOFOS8), the chimeric message is destabilized 30-fold. This region includes the sequences specified by Shaw and Kamen. When part of the 5' untranslated region of fos replaces the 5' untranslated region of globin, (GLO5 vs. FOSGLO3), the chimeric message is destabilized 2.5-fold. When the coding region of fos replaces the coding region of globin, in addition to the switch of the 5' end, (FOSGLO3 vs. FOSGLO4), there is a further 12-fold destabilizing effect. When the coding region of fos replaces the coding region of globin, in addition to the switch in the 3' end, (GLOFOS8 vs. GLOFOS7), there is a further 2.5-fold destabilizing effect. An important experiment to further assess the effects of these sequences would be to exchange the coding regions of the messages, leaving the 5' and 3' untranslated regions intact.

Mechanism:

The simplest mechanism that could confer a destabilizing or protective effect on a message would involve a small recognition sequence, mediated via the sequence itself or a local secondary structure. The sequence could signal proteins or other macromolecules to degrade or not degrade (protect) the given RNA at a given rate. However, our results suggest a more complex interpretation in which there is not one recognition motif, but several different signals up and down the extent of the RNA. Further, it could be suggested that the mechanism of recognition by the degradative pathways is mediated via a more complex secondary structure, which encompasses the 5' and the 3' ends of the RNA folded into some structure containing more extensive and inclusive secondary and/or tertiary structure. Secondary structure that

encompasses both ends of the molecule have been shown in the case of mouse β^{Maj} globin (Lockard et al., 1986) as described in Chapter IC Mechanism. Such an interaction is further implied by the results of Piechaczyk et al. (1986) in which the first exon seems to be highly destabilizing in the context of the myc message, but has no effect on the stability of the CAT transcript when a fusion gene is made. (See Chapter IA6 Mutants.) These mechanisms could work in concert with different sequences and different degradative pathways in order to degrade messages at a continuum of rates. (See Chapter IC Mechanism.) These results could be complicated by the possibility that an important sequence recognition signal could be destroyed by the arbitrary junctions of the chimeras.

Effects of Cell Confluence:

The results suggest that there was no effect of the state of confluence of the cells on half-life of these fos-promoter driven chimeric RNAs. Experiments in which cells were stimulated to produce fos at densities from 30% to 100% confluent resulted in no difference in half-life. Since data in the Chapter I suggested that there could be a quantitative difference in the stability of RNAs depending upon the state of the cells, one could argue that the observed lack of effect is idiosynchratic to cells under these particular conditions of stimulation. It is possible that quiescent cells respond in various ways to refeeding. It has been shown that the cells are not growing at all after calf serum refeeding of quiescent cells.

Translation:

The results clearly indicate that these chimeric messages were present on polysomes and hence, probably translated. Messages with the fos capping region, the globin capping region, and no obvious poly(A) signal, were all

found on monosomes and disomes at early times of induction of the fos promoter. Polysomal material was obtained from cells that had been induced for 2 hours and the chimeric message (in that case, FOSGLO3) was also detectable on polysomes. The presence of the chimeric RNAs on polysomes addresses two issues: (1) all hybrid RNAs can be considered "mRNA"s and (2) the decay curves examined in this study probably represent a single component of the RNA, since RNPs of a given species may decay at a different rate.

In the course of these experiments, a system to study preferential translation was inadvertantly created. The data show that cells made quiescent in 5% plasma for one to three days break down their polysomes and that immediately upon refeeding, the cells use the stored messages for recovering active cellular metabolism. This is seen in the performance of α-tubulin on the bottom Northern blot in Figure FOSX (Panel C) which strongly suggests that some messages were stored in RNPs. This is substantiated by Thomas and Thomas (1986) who showed that such translational control is active in Swiss mouse 3T3 cells during an identical transition from quiescence to growth. Levels of several RNAs (encoding translational elongation factor eEF-1α, poly(A) binding protein, vimentin and α- and β-tubulin) remained unchanged during this transition, though synthesis of the proteins resumed following refeeding. Some RNAs (e.g., encoding actins) do increase after refeeding. Studying the transition of messages from RNPs to monosomes, disomes and polysomes would permit detection of any preferential translatability. Differential translation between the chimeras discussed herein could still account for some or all of the differences in half-lives. This could, however, actually be part of the mechanism which determines the stability of a message. Translation and stability are intimately intertwined.

A possible artifactual result could be due to competition between messages for the potentially limited translational machinery. The idea that

the large amount of endogenous fos transcription could burden the translational apparatus, would be further compelling, given that the amount of fos-driven message would be doubled by the exogenous chimeric message. There is no evidence that the translational machinery is swamped by the extensive amount of message. Since all mRNAs are translated immediately upon induction of the message, it is doubtful that this is a problem.

Is Half-Life Dependent Upon Cellular Conditions?

Do RNA half-lives exist only in the given conditions in which they are being tested? There is some evidence which indicates that RNA stability is dependent on the cell in which it is assessed. Gurdon et al. (1973) showed that when globin RNA is injected into frog oocytes it is stable for at least two weeks, as long as the RNAs of the host cell. Maquat and Kinniburgh (1985) observed that a β^0 thalassemic globin RNA that has a half-life of 30 minutes in bone marrow cells, lasts significantly longer in HeLa cells.

The conditions in which the fos promoter is induced are specific. Interpretations may be made only in the context of these particular conditions. In our studies the conditions that stimulate the fos promoter, growth factors following a period of quiescence in plasma, probably also stimulate the interferon system. The interferon promoter was originally avoided for its direct effect in stimulating the RNase L system. The anti-viral effects of interferon are partially mediated via latent RNase L which is stimulated by (2'-5')oligoadenylate which is synthesized in response to interferon (Nilsen et al., 1982). It was shown that the anti-viral effects of interferon involved the destruction of double-stranded RNAs, including rRNA and double-stranded RNA viruses. Nilsen and Baglioni (1979) showed that double-stranded RNA covalently bound to single-stranded RNA was preferentially degraded, compared with single-stranded RNA. These authors claim that such a

mechanism may be responsible for discrimination between viral and cellular RNAs, yet it could also account for discrimination of the system between cellular RNAs that have a considerable amount of secondary structure and those that do not. Dani et al., (1985), and Jonak and Knight, (1984), claim that interferon treatement of cells results in a selective degradation of c-myc RNA. These authors suggest that the RNase L system may, in fact, act on cellular mRNAs, as well. New evidence (Zullo et al., 1985; Kohase et al., 1986, 1987) suggests that growth factors, including tumor necrosis factor, interleukin-1 and platelet-derived growth factor, all present in serum, stimulate the expression of a subset of interferon genes. These β_2-interferons are thought to exert feedback growth regulation on the stimulation supplied by the growth factors (Kohase et al., 1986). In this way, interferons, and the downstream effects of these molecules, have a greater role in cellular homeostasis than just anti-viral actions. The kinetics of this response is that the activated RNAse L persists in the cell from 2-24 hours post-serum stimulation (M. Garcia-Blanco, personal communication). Since the stimulus for the fos promoter is serum, serum contains growth factors, and growth factors have been shown to activate an RNase system via interferon (which probably can act upon cellular RNAs), it is possible that the system discussed here permits the analysis of RNA half-lives only under these specified conditions. Thus, a half-life should only be considered in the context of a given set of experimental condtions.

To determine whether the interferon-induced RNase L system was involved in changing the half-lives determined herein would be difficult. An effect on a half-life by an agent which perturbs the RNase L system would have to be considerable. To further induce the system with β_2-interferon could easily be fruitless if the system is already saturated. To inhibit the system with antagonistic analogs of (2'-5')oligoadenylate would be useless without a

potent inhibitor of which none are known. Alternatively, the use of another promoter, instead of the human c-fos promoter would prove useful for this. This would permit the study of the effects of agents that cannot be studied using the fos promoter based on how it is induced. (See description of use of lac operon system in the Conclusions.) Another promoter would permit the determination of the half-life of the fos message itself in a context in which it is not normally found. Not only would this be interesting for the dissection of the effect of environment on half-life, but also for understanding the role B2 transcripts may play in the determination of RNA stability (see Chapter IC Mechanisms).

Conclusions:

These results suggest that the subject of RNA stability is even more complex than had been previously considered. It is clear that more than just a small region of sequence or local secondary structure is involved in determining the rate at which RNA is degraded. It seems likely that the signal for the decay of human c-fos and human β-globin messages involves either many local stretches of secondary structure or the interaction between the two ends of the message. A comprehensive panel of chimeric constructs in which the coding regions of the two genes are exchanged would further elucidate the roles of untranslated regions and coding regions. From there, precise point mutations created by in vitro mutagenesis could point more specifically to crucial regions of sequences involved.

The human c-fos promoter has proven to be well-suited as a prototypical inducible promoter for use in our system designed to study determinants of RNA stability. It responds to inductive stimuli with characteristics and kinetics necessary for implementation in this system. In addition, the stimulus for activating the promoter is not toxic, though it has been shown to be involved

in effecting alterations in other cellular processes. Ideally, the agent that is employed to induce the promoter in this system would be specific, exerting no pleiotropic effect on the cell or cellular metabolism. No such highly specific inducer of a known promoter has been described to date. Given the nature of and the evolutionary purpose for an inducible promoter it is unlikely that such a promoter will be found. Were such a promoter to be discovered, it would still need to fulfil the other requirements of magnitude and kinetics of transcription for use in this system. This system is likely to be most useful for the study of RNA stability when several different promoters, each of which have little or no effect on cellular functions, are separately employed.

Figure FOSA: Construction of PUCFOS1 for the further construction of the rest of the hybrids. PCFOS itself was used as the human c-fos construct which was transfected. Pertinent sites are as marked on the plasmids. The sections marked "coding" stretch from the cap to the end of the message and include introns. Arrow a: PCFOS (Curran et al., 1983, Van Straaten et al., 1983) was cut with Xho1 and then recut with Nsi1. The 3.9 kb fragment containing all of the fos promoter region and most of the fos gene was gel isolated. Arrow b: PUC19 was cut with Sal1 and then with Pst1 and the vector was purified on a gel. Arrow c: Two fragments were ligated at 20 μg/ml for 3 hours at 15°C to allow the sticky ends to anneal and ligate (Xho1 and Sal1 leave compatible, cohesive ends, as do Nsi1 and Pst1. The sites for the exzymes are destroyed.) The ligation was transformed into HB101 and plated onto L-B agar with 50 μg/ml ampicillin. Arched lines demarcate the origin of different regions of DNA in the plasmid.

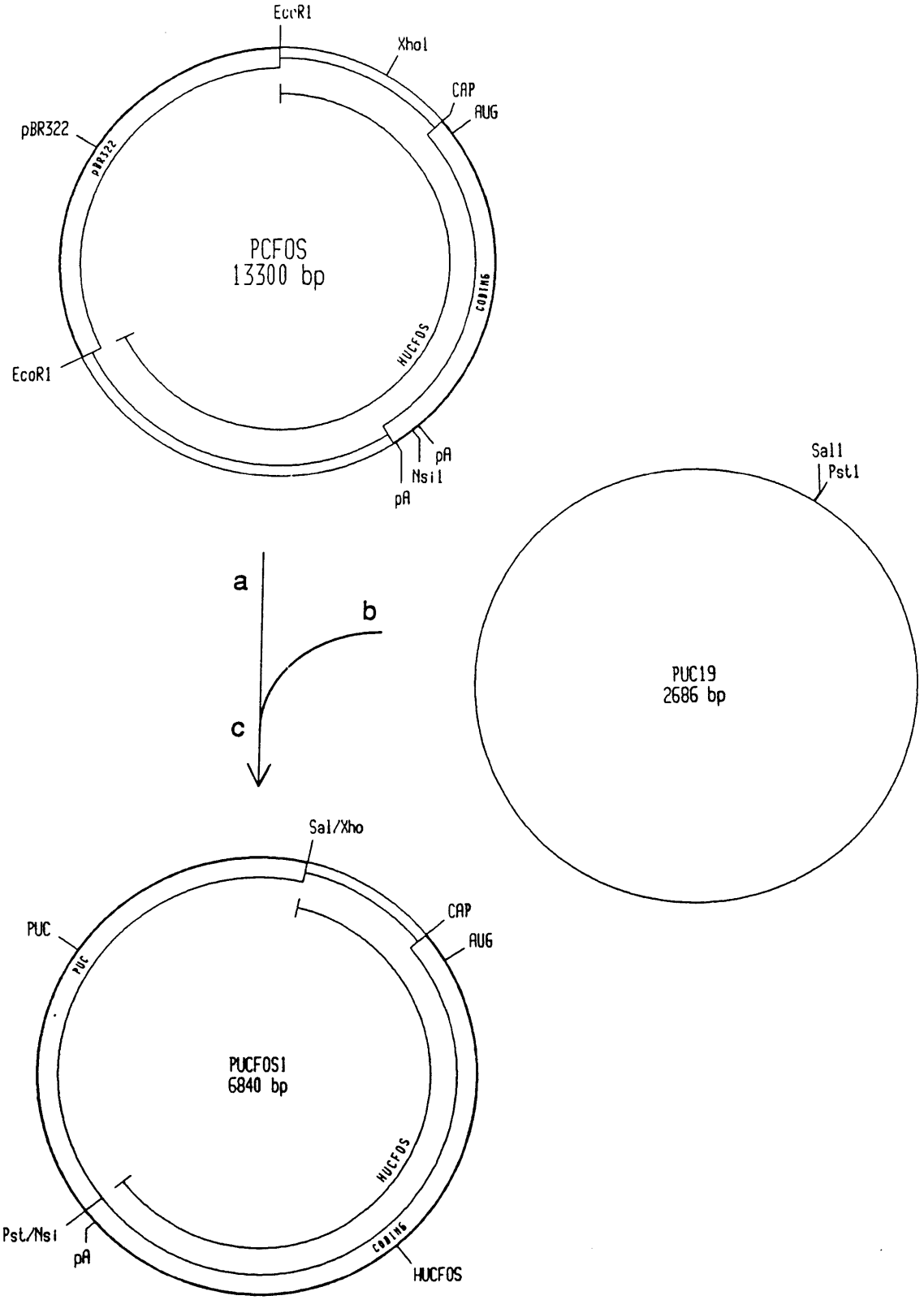

EcoR1
Xho1
CAP
AUG
pBR322
PCFOS
13300 bp
CODING
HUCFOS
EcoR1
pA
Nsi1
pA
a
b
c
Sal1
Pst1
PUC19
2686 bp
Sal/Xho
CAP
AUG
PUC
PUC
PUCFOS1
6840 bp
HUCFOS
Pst/Nsi
pA
CODING
HUCFOS

Figure FOSB: Construction of FOSGLO3. Pertinent sites are as marked on the plasmids. The sections marked "coding" stretch from the cap to the end of the message and include introns. Arrow a: PUCFOS1 was cut with Nae1 and then recut with Hind3. The 3.4 kb fragment containing PUC, the fos promoter and fos 5' leader sequence (from NaeA to Hind3) was isolated from a gel. Arrow b: HUBGLO was cut with Nco1, blunted by treatment with Klenow fragment and recut with Hind3. The 3.13 kb piece containing all of the coding region of globin and about 750 bases of pBR322 was gel isolated. Arrow c: Two fragments were ligated at 20 μg/ml for 3 hours at 15^{o}C to ligate the cohesive Hind3 ends and then diluted 10-fold and incubated overnight at 30^{o}C to permit blunt ends to ligate. Transformation was as described in legend to Figure FOSA. Arched lines demarcate the origin of different regions of DNA in the plasmid.

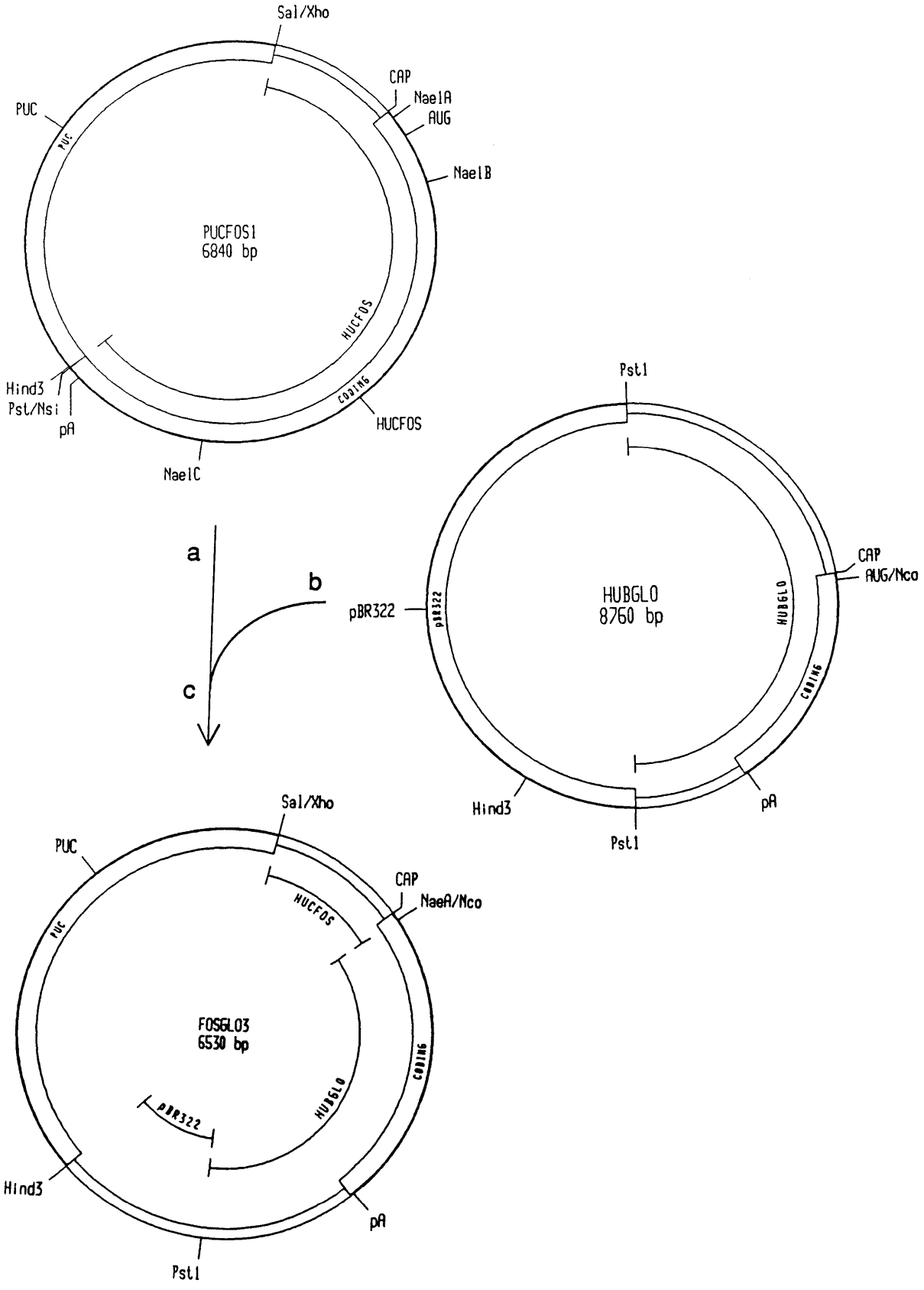

Sal/Xho
CAP
NaeIA
AUG
NaeIB
PUC
PUCFOS1
6840 bp
HUCFOS
CODING
HUCFOS
Hind3
Pst/Nsi
pA
NaeIC
a
b
c
Pst1
CAP
AUG/Nco
pBR322
HUBGLO
8760 bp
HUBGLO
CODING
Hind3
pA
Pst1
Sal/Xho
PUC
CAP
NaeA/Nco
HUCFOS
FOSGLO3
6530 bp
HUBGLO
CODING
pBR322
Hind3
pA
Pst1

Figure FOSC: Construction of FOSGLO4. Pertinent sites are as marked on the plasmids. The sections marked "coding" stretch from the cap to the end of the message and include introns. Arrow a: PUCFOS1 was cut partially in the presence of 50 μg/ml ethidium bromide with .5 u/μg plasmid DNA with Ssp1 for 30 minutes. (The concentration of ethidium bromide used was determined by its titration from 1 to 200 μg/ml under similar conditions to maximize for single cut plasmids (Parker et al., 1976).) Linear, single cut DNA was isolated from a gel and recut with Hind3. The 6.44 kb piece from Ssp1A to Hind3 was purified on a gel. Arrow b: HUBGLO was cut with BstX1, blunted with T4 polymerase and recut with Hind3. The 1.8 kb fragment containing the 3' end of globin and a small piece of pBR322 was gel isolated. Arrow c: The two fragments were ligated and transformed as described in the legend to Figure FOSB. Arched lines demarcate the origin of different regions of DNA in the plasmid.

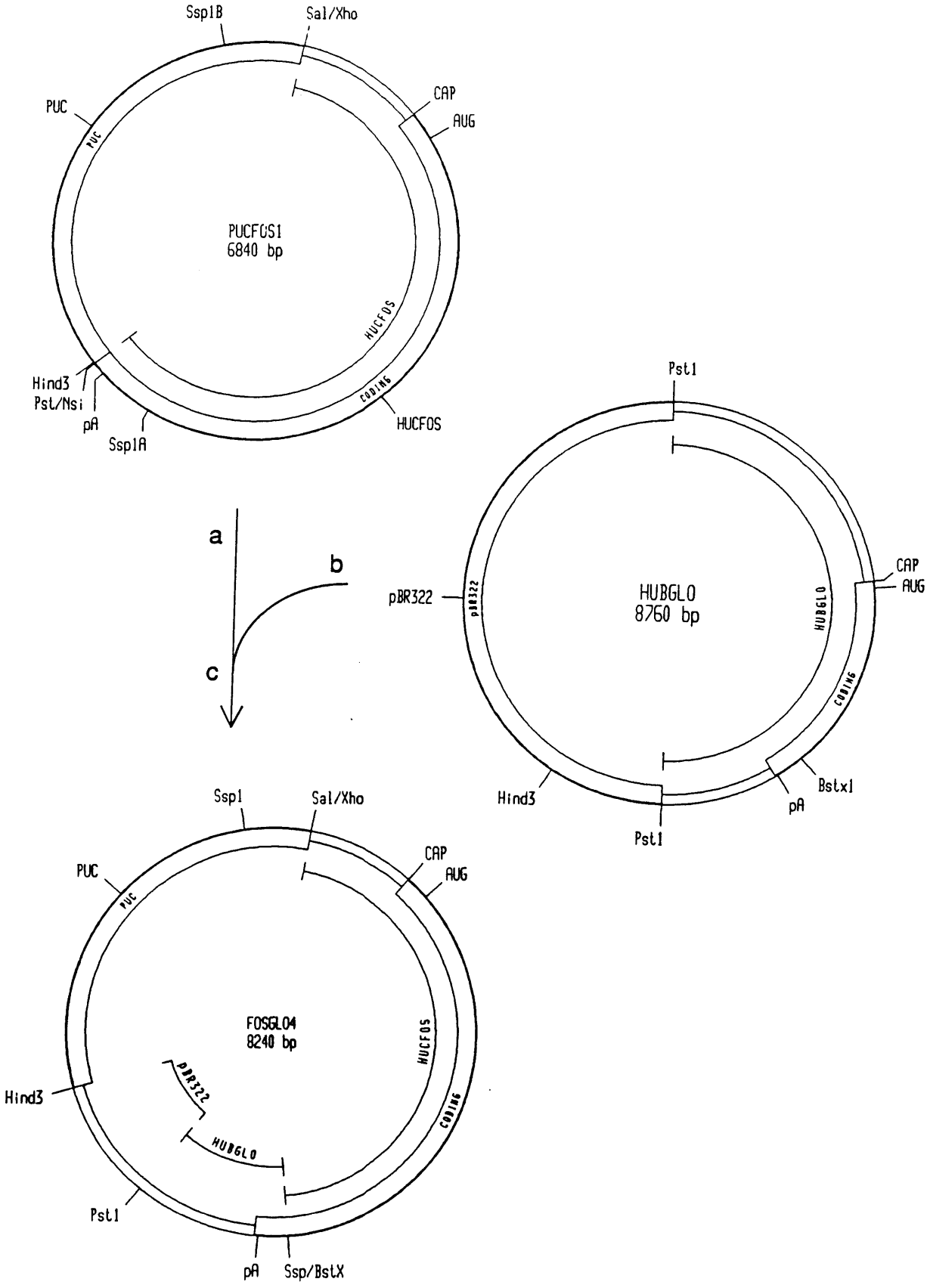

Ssp1B
Sal/Xho
CAP
AUG
PUC
PUC
PUCFOS1
6840 bp
HUCFOS
CODING
Hind3
Pst/Nsi
pA
Ssp1A
HUCFOS
a
b
c
Pst1
pBR322
pBR322
HUBGLO
8760 bp
HUBGLO
CODING
CAP
AUG
Hind3
pA
Bstx1
Pst1
Ssp1
Sal/Xho
CAP
AUG
PUC
PUC
FOSGLO4
8240 bp
HUCFOS
CODING
pBR322
HUBGLO
Hind3
Pst1
pA
Ssp/BstX

Figure FOSD: Construction of FOS6. Pertinents sites are as marked on the plasmids. The sections marked "coding" stretch from the cap to the end of the message and include introns. Arrow indicates that PUCFOS1 was isolated as in arrow a of Figure FOSC. The purified piece was blunted by treatment with Klenow fragment and ligated to itself at 1 μg/ml overnight at 30^{o}C. Transformation was performed as described in legend to Figure FOSA. Arched lines indicate the origin of different regions of DNA in the plasmid.

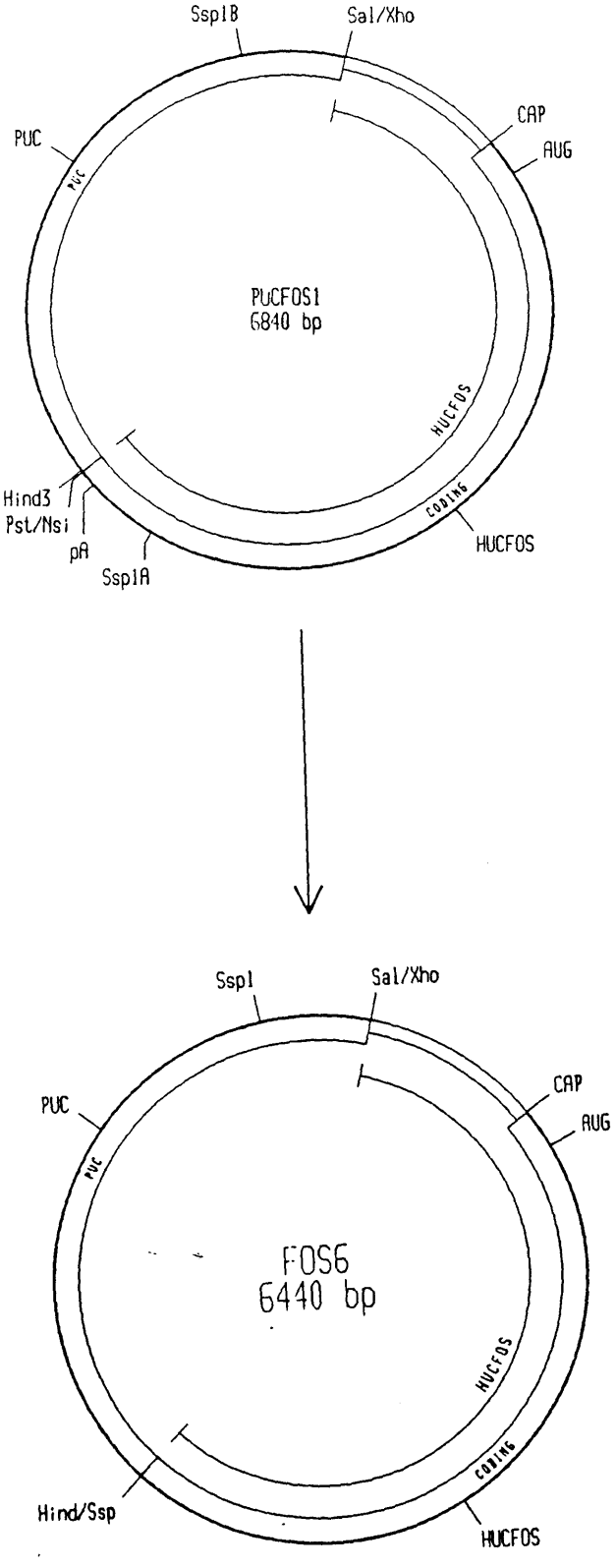

SspIB
Sal/Xho
CAP
AUG
PUC
PUC
PUCFOS1
6840 bp
HUCFOS
CODING
HUCFOS
Hind3
Pst/Nsi
pA
SspIA
SspI
Sal/Xho
CAP
AUG
PUC
PUC
FOS6
6440 bp
HUCFOS
CODING
HUCFOS
Hind/Ssp

Figure FOSE: Construction of M13MTGLO for the eventual construction of M13FOSGLO for the construction of GLO5 and derivative plasmids. The promoter-message start junction of NEOMTGLO had been placed in M13 as the "perfect" globin message was going to be made from the metallothionein promoter before that promoter was dropped. Pertinents sites are as marked on the plasmids. Arrow a: M13MP19 was cut with Hind3 and then BamH1 and then gel isolated. Arrow b: NEOMTGLO (see Figure MTC) was cut with the same enzymes and the 2.6 kb fragment containing the MT promoter and the 5' end of globin was gel purified. Arrow c: The fragments were ligated and the ligation transformed as described in the legend to Figure FOSA. Arched arrows indicate the extent of transcription.

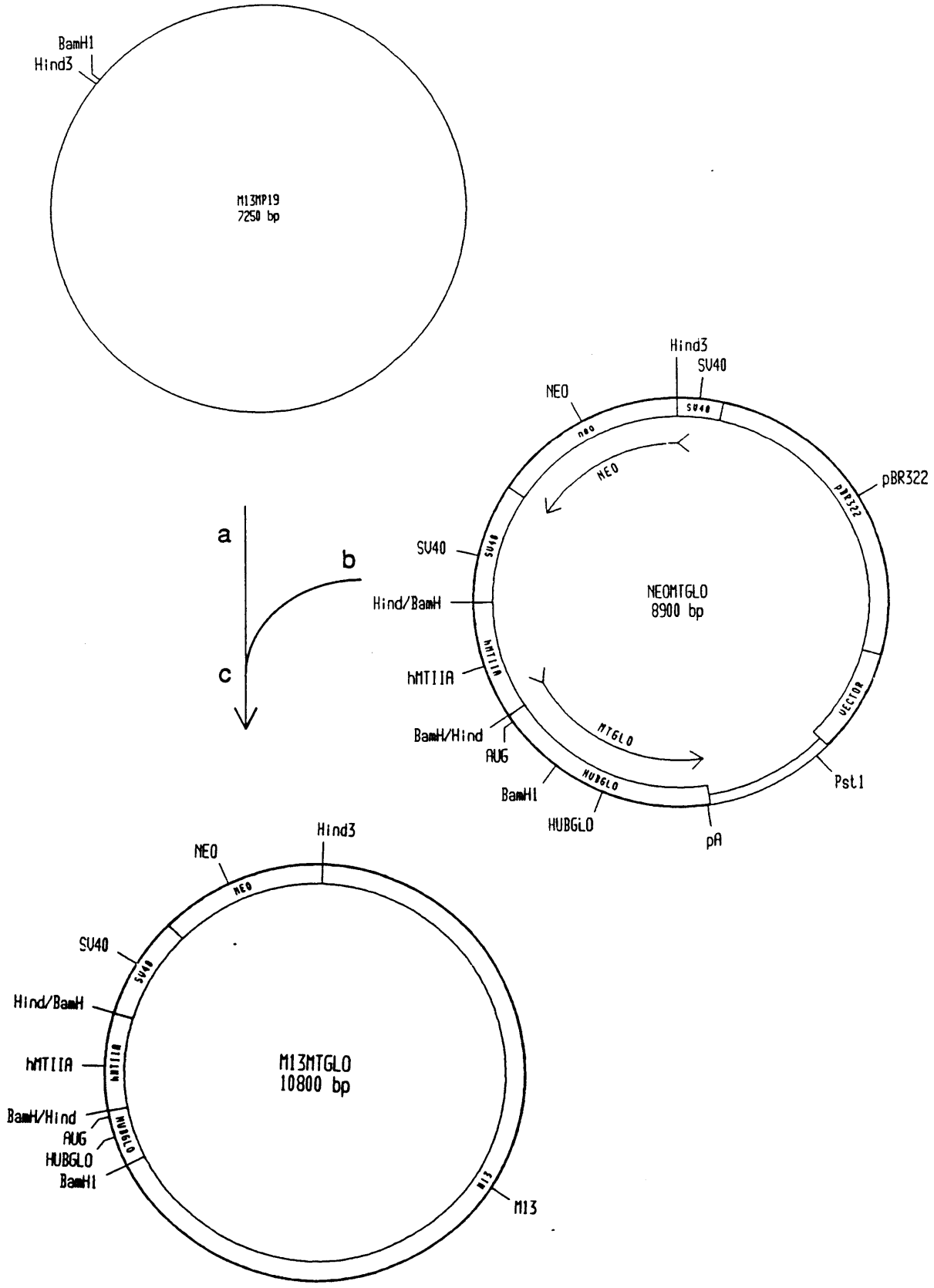
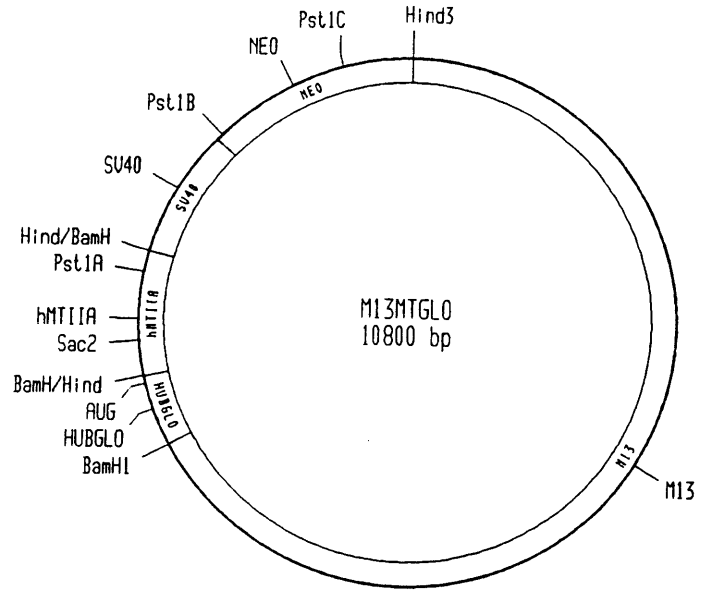
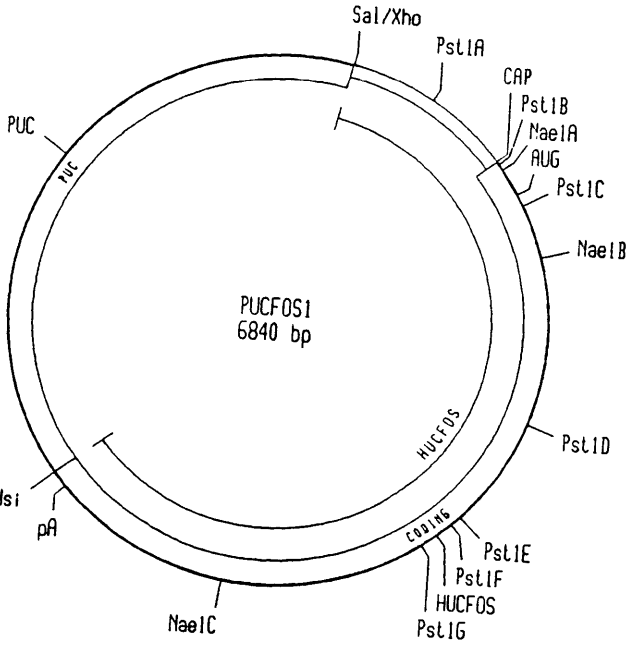
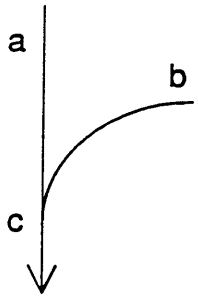

BamH1
Hind3
M13MP19
7250 bp
Hind3
SV40
NEO
pBR322
NEOMTGLO
8900 bp
SV40
Hind/BamH
hMTIIA
BamH/Hind
AUG
BamH1
HUBGLO
pA
Pst1
a
b
c
NEO
Hind3
SV40
Hind/BamH
hMTIIA
BamH/Hind
AUG
HUBGLO
BamH1
M13MTGLO
10800 bp
M13

Figure FOSF: Construction of M13MTFOSGLO for the creation of M13FOSGLO. Pertinent sites are as marked on the plasmids. The sections marked "coding" stretch from the cap to the end of the message and include introns. Arrow a: M13MTGLO was cut with Sac2 and then with Pst1 to completion. 8.56 kb DNA was isolated on a gel. Arrow b: PUCFOS1 was cut to completion with Nae1. The larger fragment from NaeC to NaeA (3.98 kb) containing PUC and the 5' end of fos was gel purified. It was then partially recut with Pst1 and the .4 kb fragment from Pst1A to NaeA was isolated on a gel. Arrow c: Ligation and transformation was performed as described in legend of Figure FOSA. Arched arrows indicate the extent of transcription. Arched lines demarcate the origin of different DNAs in the plasmid.


Pst1C
Hind3
NEO
Pst1B
SV40
Hind/BamH
Pst1A
hMTIIA
Sac2
BamH/Hind
AUG
HUBGLO
BamH1
M13MTGLO
10800 bp
M13


Sal/Xho
Pst1A
CAP
Pst1B
Nae1A
AUG
Pst1C
Nae1B
PUC
PUCFOS1
6840 bp
Pst1D
Pst/Nsi
pA
Pst1E
Pst1F
HUCFOS
Pst1G
Nae1C


a
b
c

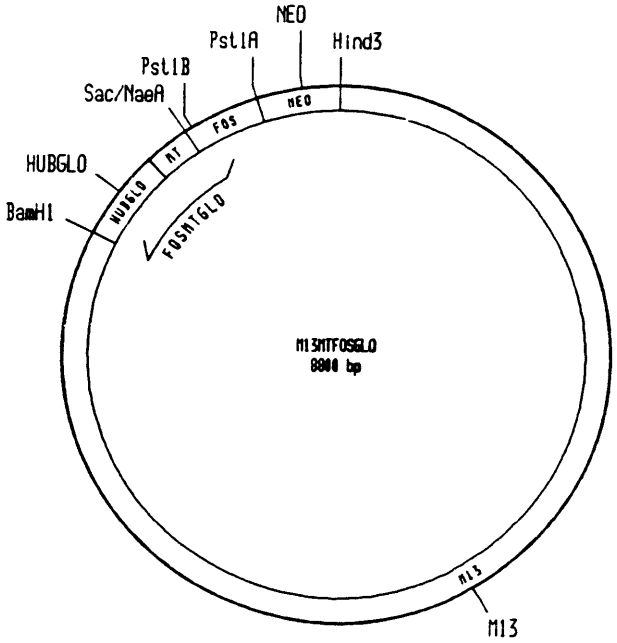

NEO
Pst1A
Hind3
Pst1B
Sac/NaeA
HUBGLO
BamH1
FOSMTGLO
M13MTFOSGLO
8800 bp
M13

Figure FOSG: Construction of M13FOSGLO. Pertinent sites are as indicated in the plasmids. Arrow a: The region of M13MTFOSGLO marked by the arched line is expanded. The upper line indicates the sense strand of that region. The lower line is an 25-mer that was made which exactly spans the region of GLO, MT and FOS which is to be deleted in arrow b. The junction was created so as to be from -16 to -4 of FOS connected to -4 to +8 of GLO. The manipulations in arrow b cause the DNA between the ends of the mutagenic oligo to loop out and be destroyed upon transformation. Arrow b: Single stranded M13MTFOSGLO DNA was purified and annealed to the mutagenic oligo described above, which was kinased, and to a universal M13 priming oligo. The DNA was then incubated overnight at 15^{o}C with Klenow fragment to extend the second strand started by the oligos and with T4 DNA ligase to covalently bond the ends. This extension/ligation reaction was then transformed into competent JM101, supplemented with exponentially growing JM101 and plated using soft agar. 48 M13 plaques were grown up as minipreps, the supernatant saved as a virus stock, the bacteria treated with normal miniprep procedures to isolate the RF form of each virus. The DNAs were first screened by cutting with Pst1, looking for the deletion of Pst1B. 14 of these had lost the Pst1B site and were further checked by fine restriction mapping on acrylamide gels. The one version which was used for the construction of GLO5 (Figure FOSH) and derivative vectors was sequenced and shown to be exactly as expected. Arched arrows indicate extent of transcription. Arched lines demarcate the origin of DNA in the plasmid.

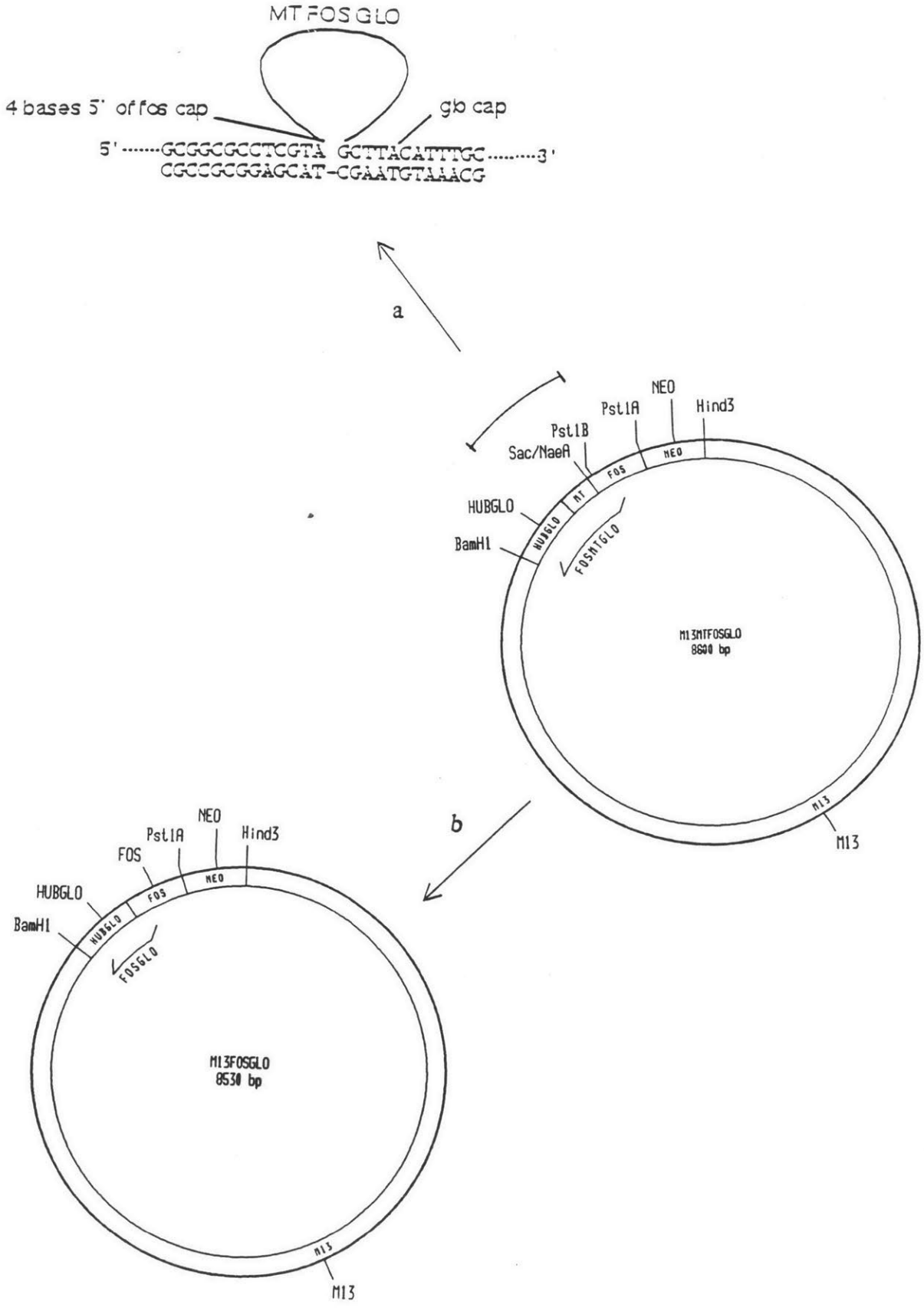

MTFOSGLO
4 bases 5' of fos cap
glb cap
5'.....GCGGCGCCTCGTA GCTTACATTTGC.....3'
CGCCGCGGAGCAT-CGAATGTAAACG
a
NEO
Pst1A
Hind3
Pst1B
Sac/NaeA
FOS
MT
HUBGLO
HUBGLO
BamH1
FOSMTGLO
M13MTFOSGLO
8600 bp
M13
M13
b
NEO
Pst1A
Hind3
FOS
HUBGLO
FOS
BamH1
HUBGLO
FOSGLO
M13FOSGLO
8530 bp
M13
M13

Figure FOSH: Construction of GLO5. Pertinent sites are as marked on the plasmids. The sections Marked "coding" stretch from the cap to the end of the message and include introns. Arrow a: M13FOSGLO was cut with Apa1 and then BamH1. The .7 kb piece containing the FOSGLO junction described in legend to Figure FOSG was gel isolated. Arrow b: FOSGLO3 (Figure FOSB) was partially cut with BamH1 using ethidium bromide as described in legend to Figure FOSC except that 75 μg/ml ethidium was used due to the titration results. The DNA was recut with Apa1 and the 5.42 kb fragment from BamH1A to Apa1 containing most of GLO, all of PUC and the 5' end of FOS was isolated. Arrow c: the two DNAs were ligated and transformed as described in the legend to Figure FOSB. Arched lines indicate the origin of DNA in the plasmid.

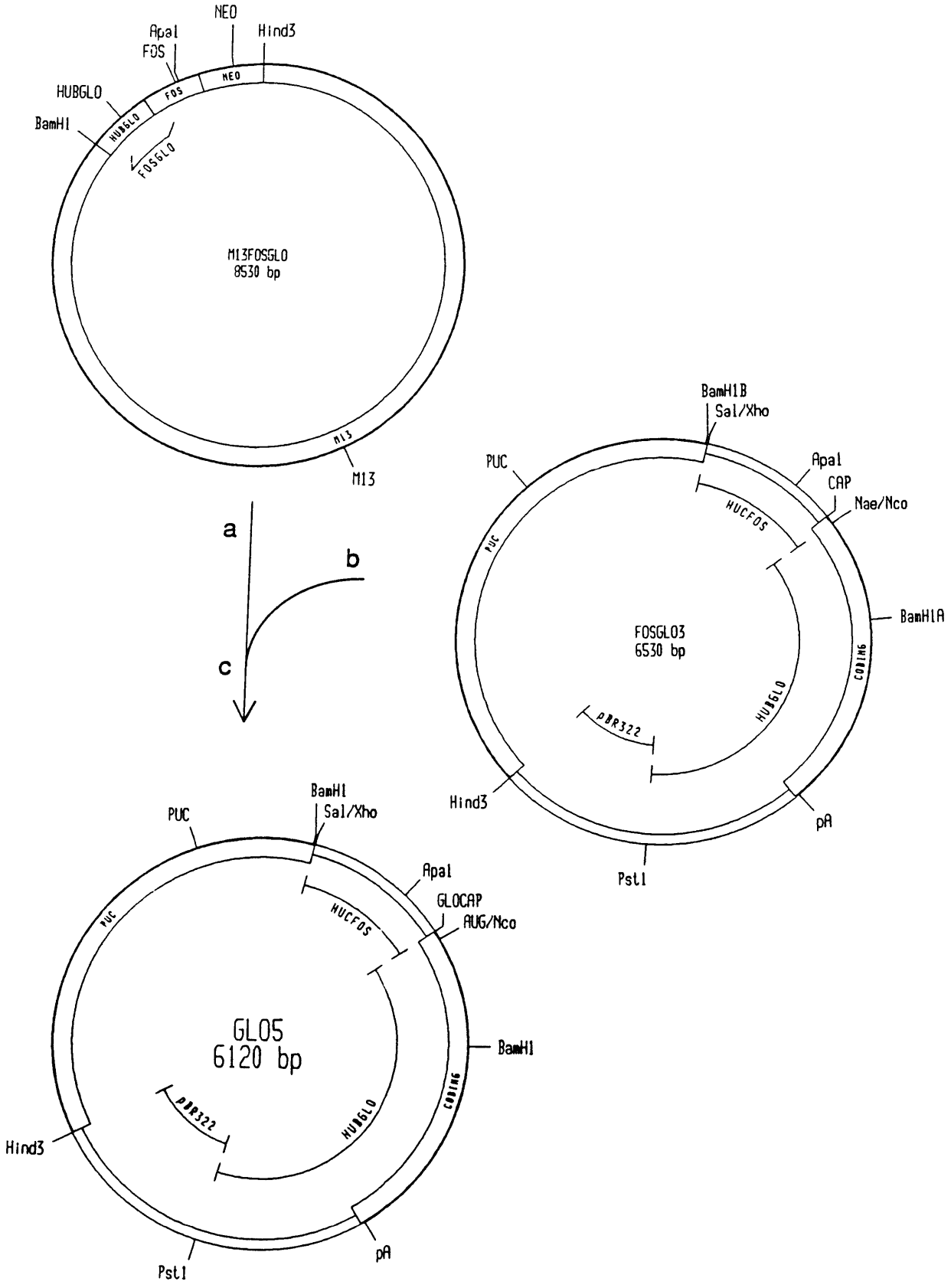

NEO
ApaI
FOS
Hind3
HUBGLO
BamHI
FOS
NEO
HUBGLO
FOSGLO
M13FOSGLO
8530 bp
M13
M13
a
b
c
BamHIB
Sal/Xho
PUC
ApaI
CAP
Nae/Nco
HUCFOS
PUC
BamHIA
FOSGLO3
6530 bp
CODING
pBR322
HUBGLO
Hind3
pA
PstI
BamHI
Sal/Xho
PUC
ApaI
GLOCAP
AUG/Nco
HUCFOS
PUC
BamHI
GLO5
6120 bp
CODING
pBR322
HUBGLO
Hind3
pA
PstI

Figure FOSI: Construction of PUCFOS10 for the creation of constructs GLOFOS7 and GLOFOS8 to contain the entire 3' sequences of fos. Pertinent sites are marked on the plasmids. The sections marked "coding" stretch from the cap to the end of the message and include introns. Arrow a indicates that PCFOS was cut with Hind3 to completion and then with XhoI. The 5.2 kb fragment from XhoI to Hind3A was purified on a gel. Arrow b: PUC19 was cut with Hind3 and then SalI. It was isolated on a gel. Arrow c: Since Xho1 and Sal1 have compatible ends the ligation and transformation was performed as in legend to Figure A. Arched lines indicate the origin of DNA in the plasmid.

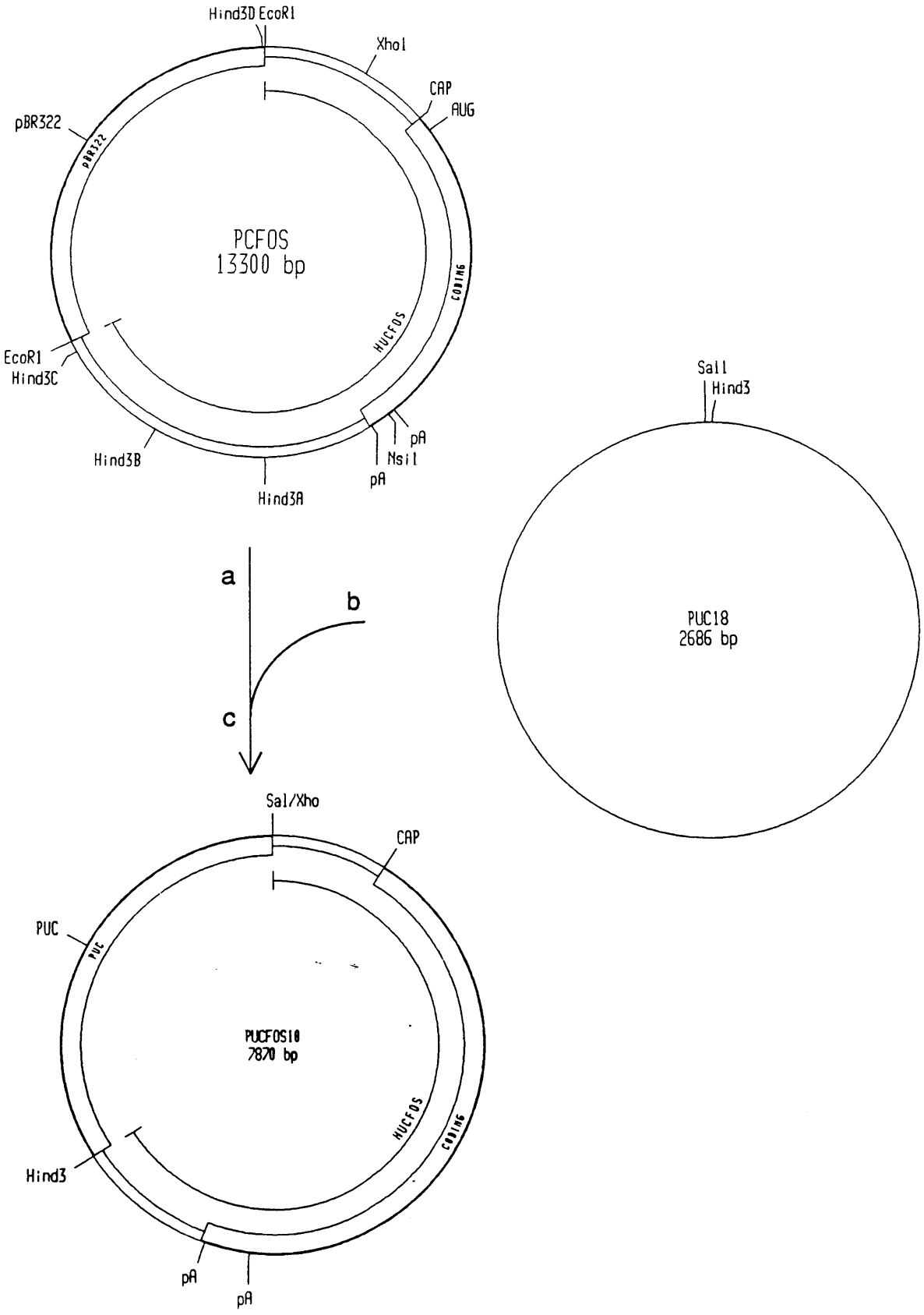

Hind3D EcoR1
XhoI
CAP
AUG
pBR322
pBR322
PCFOS
13300 bp
CODING
HUCFOS
EcoR1
Hind3C
Hind3B
Hind3A
pA
NsiI
pA
a
b
c
Sal/Xho
CAP
PUC
PUC
PUCFOS10
7870 bp
HUCFOS
CODING
Hind3
pA
pA
SalI
Hind3
PUC18
2686 bp

Figure FOSJ: Construction of GLOFOS7. Pertinent sites are marked on the plasmids. The sections marked "coding" stretch from the cap to the end of the message and include introns. Arrow a: GLO5 was cut with Nco1, blunted with Klenow fragment and then recut with Hind3. 3.26 kb fragment containing PUC, FOS promoter and the 5' leader of globin was gel isolated. Arrow b: PUCFOS10 was cut partially using ethidium bromide as described in the legend to Figure FOSC except that 50 μg/ml ethidium bromide was used. Single cut, linears were gel purified and cut to completion with Hind3. The 4.4 kb piece of DNA containing most of FOS was purified on a gel. Arrow c: Ligation and transformation were performed as described in legend of Figure FOSB. Arched lined indicate the origin of DNA in the plasmid.

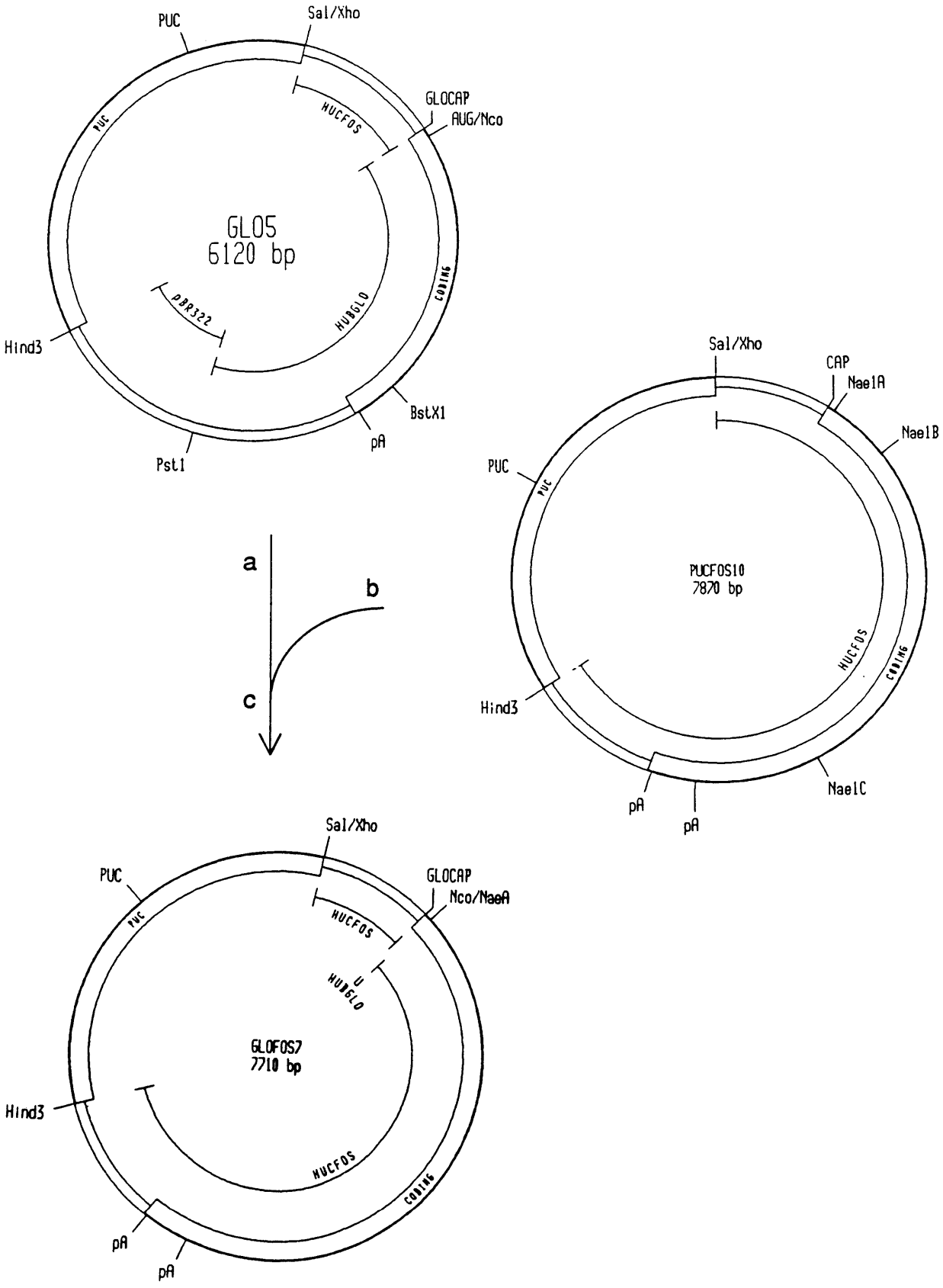

PUC
Sal/Xho
GLOCAP
AUG/Nco
HUCFOS
PUC
GL05
6120 bp
pBR322
HUBGLO
CODING
Hind3
BstX1
pA
Pst1
a
b
c
Sal/Xho
CAP
NaeIA
NaeIB
PUC
PUC
PUCFOS10
7870 bp
HUCFOS
CODING
Hind3
pA
pA
NaeIC
Sal/Xho
PUC
GLOCAP
Nco/NaeA
PUC
HUCFOS
U
HUBGLO
GLOFOS7
7710 bp
Hind3
HUCFOS
CODING
pA
pA

Figure FOSK: Construction of GLOFOS8. Pertinents sites are as marked on the plasmids. The sections marked "coding" stretch from the cap to the end of the message and include introns. Arrow a: GLO5 was cut with BstX1, blunted with T4 polymerase and recut with Hind3. The 4.3 kb fragment containing most of globin, fos promoter and PUC was gel isolated. Arrow b: PUCFOS10 was cut partially with Ssp1 using ethidium bromide as described in the legend to Figure FOSC except that 50 μg/ml ethidium bromide was used. Purified linears were recut with Hind3 and the 1.4 kb piece of DNA from Ssp1A to Hind3 containing the 3' end of FOS was isolated. Arrow c: Ligation and transformation were performed as in the legend to Figure FOSB. Arched lines indicate the origin of DNA in the plasmid.

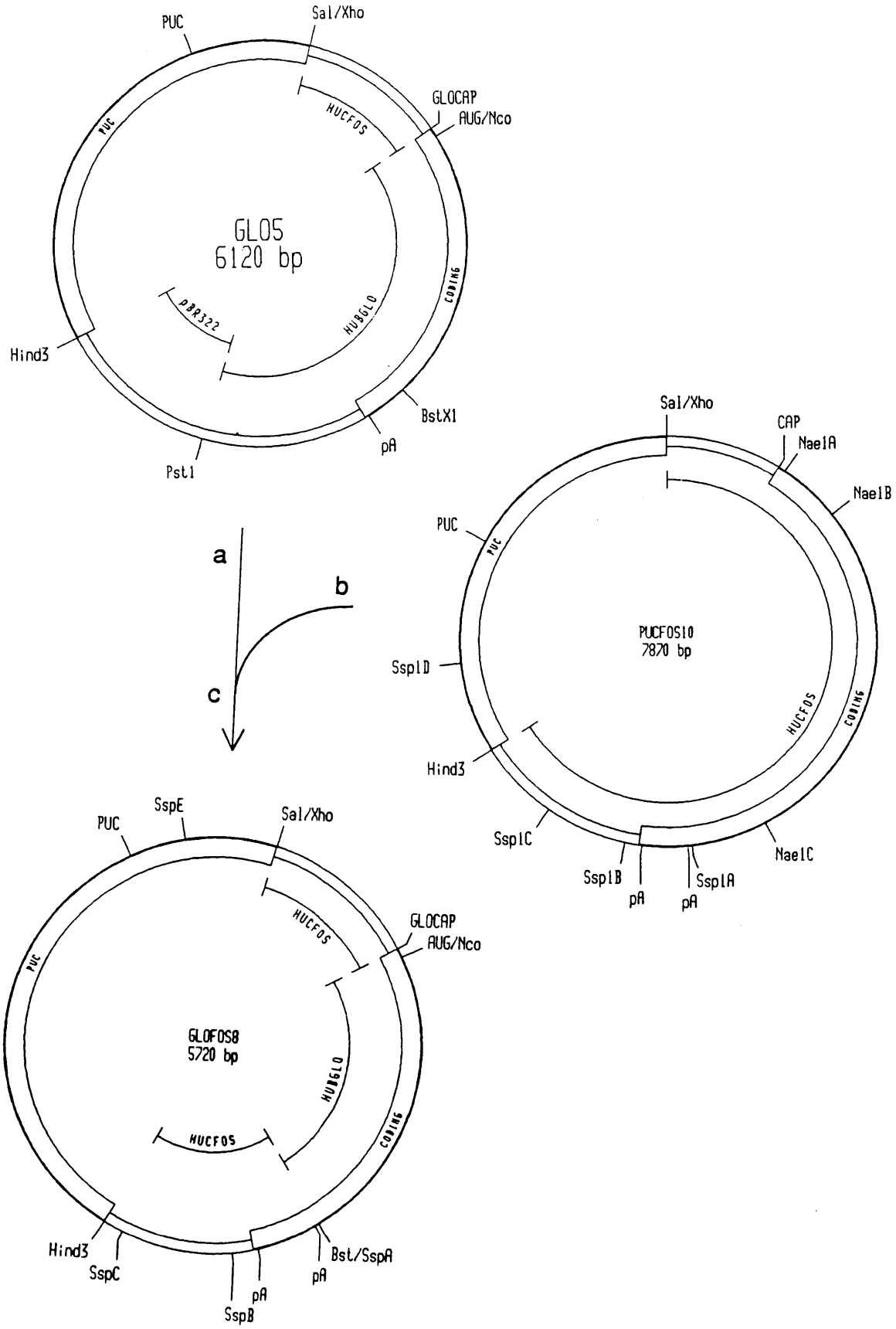
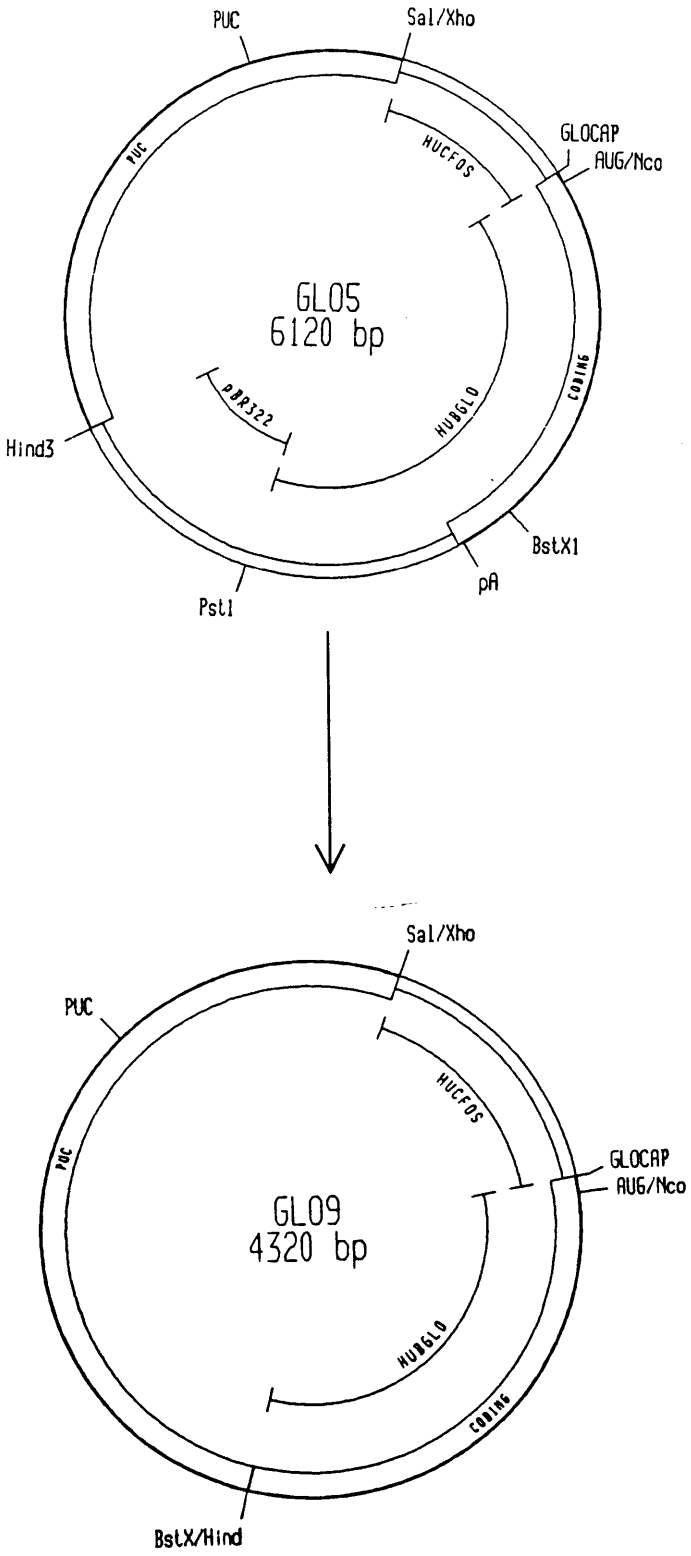

PUC
Sal/Xho
GLOCAP
AUG/Nco
HUCFOS
PUC
GLO5
6120 bp
pBR322
HUBGLO
CODING
Hind3
BstX1
pA
Pst1
a
b
c
Sal/Xho
CAP
NaeIA
NaeIB
PUC
PUC
PUCFOS10
7870 bp
SspID
HUCFOS
CODING
Hind3
SspIC
NaeIC
SspIB
SspIA
pA
pA
PUC
SspE
Sal/Xho
HUCFOS
GLOCAP
AUG/Nco
PUC
GLOFOS8
5720 bp
HUBGLO
HUCFOS
CODING
Hind3
SspC
SspB
pA
pA
Bst/SspA

Figure FOSL: Construction of GLO9. Pertinent sites are as marked on the plasmids. The sections marked "coding" stretch from the cap to the end of the message and include introns. Arrow indicates that GLO5 was cut and purified as in the legend to Figure FOSK. It was then blunted with Klenow fragment and circularized and transformed as described in the legend to Figure FOSD. Arhced lines indicate the origin of DNA in the plasmid.


PUC
Sal/Xho
GLOCAP
AUG/Nco
HUCFOS
PUC
GL05
6120 bp
pBR322
HUBGLO
CODING
Hind3
BstX1
pA
Pst1
Sal/Xho
PUC
HUCFOS
PUC
GLOCAP
AUG/Nco
GL09
4320 bp
HUBGLO
CODING
BstX/Hind

Chapter V

Conclusions

Conclusions

These experiments provide a new approach to the study of RNA stability. The system is now primed to investigate specific sequences that could be involved in signalling RNA degradation. Any message could be placed in this system. Of immediate interest would be to assess the stabilities of δ- and β-globin and chimeras between the two, as Ross and Pizarro (1983) have shown that there is a four-fold difference in the half-lives of these messages in human nucleated bone marrow cells. Finely mapped chimeras would define specific differences which could account for the differences in half-lives. Other messages of particular interest to study in this system are the "competence" genes, including myc, which are actually induced under these circumstances. In this way, no unexpected treatment will befall these RNAs. Once several of these sites have been localized along the length of many messages, computer comparative analysis would permit the detection of regions common to particular RNAs. Mutants could be formed via site-directed mutagenesis and these mutants could be studied to further localize and define the signalling regions. Very long-lived messages might be problematic in light of the high level of constitutive expression from this promoter with construct GLO5.

Once in vivo sites of importance have been noted in this system, the chimeras and/or mutagenized RNAs along with their wild-type counterparts could then be utilized to study proteins and/or other macromolecules that are involved in carrying out the function of these signals, that is, what molecules mediate the effects of these signals. Once placed in an SP6 system, transcripts created in vitro could then be incubated with cytoplasmic extracts in order to isolate proteins (Konarska and Sharp, 1986) directly responsible for the degradation or signalling the

degradation of the RNA. Proteins that bind wild-type and not mutant RNAs would be candidates. Another approach would be to run cytoplasmic extracts on an SDS/polyacrylamide gel, transfer the gel to a filter membrane and probe it separately with both mutant and wild-type radiolabeled RNA, expecting a different pattern of binding for proteins which are involved in this trigger (Gerke and Steitz, 1986; Tazi et al., 1986).

Further in vivo studies would provide information about the effects of various perturbations on message stability. Questions raised herein could be explored in more depth. Is there an effect of the state of confluence of the cells on the half-lives of any message? Can the system be manipulated to determine the effect, if any, of growth rate on message stability? Is the interferon-induced RNase system involved in changing the stability of certain messages under certain circumstances? Attempts are being made to synthesize potent agonists of (2'-5')oligoadenylate in hopes of mimicking interferon's anti-viral effects with drugs. Antagonists may result from this research, which may be potent enough to block a significant portion of (2'-5')oligoadenylate action to see whether blocking the action of this RNase L inducer increases the stability of any message. Noxious treatments (heat shock and $ZnCl_2$) could be used in this well-controlled system to assess any real effect on stability. In addition, any difference in the half-lives of poly(A)+ and poly(A)- RNA could easily be addressed.

Another inducible promoter would prove useful in assessing the stabilities of these and other messages under different conditions. Recently, another potentially useful system, the lac system, has appeared in the literature (Hu and Davidson, 1987; Brown et al., 1987). It has been shown to exert control over the expression of heterologous DNA in mammalian cells. Cells were transfected with E. coli DNA containing the lac

operator-repressor system in which the lac repressor was constitutively produced and bound to the lac operator, located downstream of either the SV40 promoter (Brown et al., 1987) or the RSV-LTR (Hu and Davidson, 1987). When repressor is bound the promoter is non-functional. For induction, isopropyl β-D-thiogalactoside (IPTG) is added to the media to bind lac repressor, decreasing its affinity for the operator region and hence, derepressing the promoter. This system has the advantage that it is apparently neutral and harmless with respect to its inducer. However, it is possible that IPTG is not as harmless as thought in mammalian cells. Moreover, the kinetics and magnitude of the transcriptional induction may not be rapid useful for these studies. Hu and Davidson (1987) have shown that CAT activity driven by this derepression continued to increase up to at least 48 hours in the presence of IPTG. The first time point of CAT activity shown was at 12 hours in the presence of IPTG, a point at which not much activity was detected. Titration of inducer and time courses in the presence and removal of inducer could reveal kinetics that are useful for the study of RNA stability, but at this point it is useless.

The fos induction protocol that we have developed alleviates many of the technical problems associated with the measurement of RNA stability. The application and extension of this approach to identify determinants of RNA stability should, therefore, be a significant contribution to the understanding of this important issue.

Chapter VI

Appendix

Quantitation and Statistical Analyses for Half-life Determination

Quantitation and Statistic Analyses for Half-life Determination

Densitometry:

Quantitative measurements of the single bands on the Northern blots were made using an LKB Ultroscan Scanning Densitometer. All evaluations of band intensities were made on autoradiographs whose film, XAR 5, was linearized prior to exposure to the blot. Such linearization was accomplished by exposing the film to a flash of light whose intensity increased the optical density of the film roughly .15 O.D. units at 540 nm relative to unflashed film (Laskey and Mills, 1977). Such a procedure ensures that the film is in the linear range such that a longer exposure of the blot increases the intensity of the resultant band by the same factor as the increase in time of the exposure. This is required for meaningful quantitation. Each blot with a given probe was exposed several times to make certain that the film was, in fact, in the linear range. An exposure was only used for quantitation if a longer exposure of that blot gave that same fold higher densitometric intensity as the increase in time of exposure. Exposures were done at room temperature or at -80^{o}C with a Kronex intensifying screen depending upon the strength of the signal.

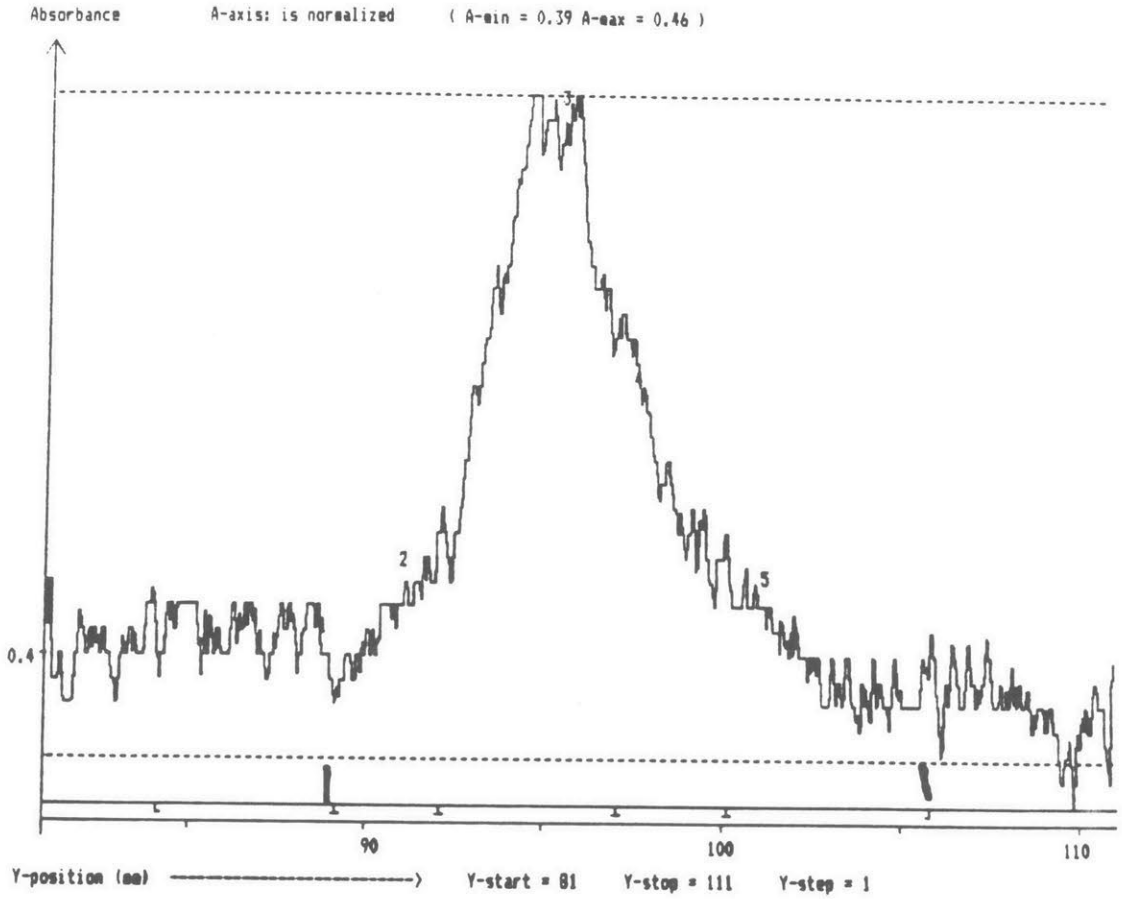
The densitometric analysis was performed on the LKB densitometer which uses a Helium/neon laser beam at 633 nm. The following parameters were set. The baseline was set by the average of the 16 lowest data points. A single linear laser beam scanned each lane 3 times in a region that seemed uniform as determined by human eye. The output scan was an average of the 3 scans, each 800 μm wide, once exactly on the set position, once to its right and once to its left. Peak limits were assigned by the densitometer and noted by small upside-down T's, upside-down L's and upside-down,

backward L's just beneath the output scan. The absorbance axis was normalized such that the single peak in each lane filled the output box, regardless of the actual maximal absorbance. This allowed the shape of the peak to be used more easily for assigning the limits of the peak if at all ambiguous, as in cases in which the total absorbance is quite low (Figure APA). Peak width was set at 9, a relative value that empirically gave me useful peak assignments and limits, in most cases. Peak areas, the values used as Northern blot band intensities, were provided by the LKB unit in a table below the printed scan. In some cases the peak delimitations were unexpected. In these scans several peaks' absorbances were pooled (Figure APA). In most cases, however, the single peak was properly bounded (Figure APB). In a few cases, the scan could not be used if the peak limits set by the densitometer seemed to be unable to interpret the peak in a human manner (Figure APC). In these cases the film was rescanned using a larger range of the film, starting further below the band and ending further above the band.

Quantitative Analysis:

The simplest model for decay of RNA is that of monotonic disappearance of the message. Such a model invokes first order kinetics, in that the degradation of a given RNA is dependent only upon the concentration of that RNA species. All half-lives determined herein fit this model of decay or else were discarded based on statistical criteria, as described below. In these cases, the data did not fit any other model better. A species of RNA containing two component subspecies which decay at different rates would better fit a biphasic curve. No data fit such a curve. This could be explained by the fact that the time courses viewed obscured any second component or that no second component existed. A convenient unit of decay

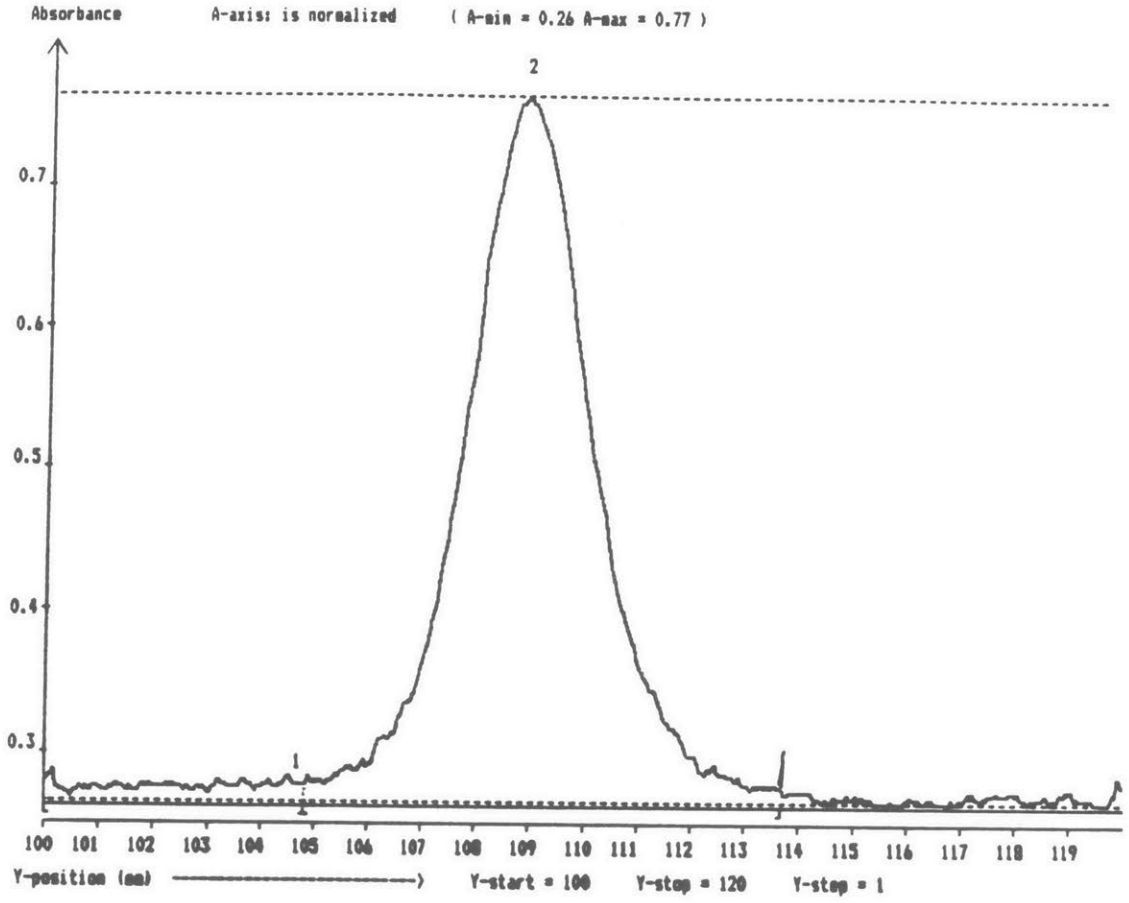
Figure APA: A densitometric scan of a low intensity band. A low intensity band of a Northern blot was scanned by the LKB Densitometer. The scan is normalized so as to fill the output box. Parameters have been set as detailed in the text. Peak limits are assigned by the densitometer as described in the text. The absorbance is the total of all 4 peaks listed in the table below.



PEAK #	POSITION mm	HEIGHT AU	AREA AU*mm	REL. AREA %	PEAK #	POSITION mm	HEIGHT AU	AREA AU*mm	REL. AREA %
1	88.8	0.01	0.06	12.8	4	97.8	0.03	0.09	18.2
2	91.1	0.02	0.04	8.5	5	101.1	0.02	0.06	11.4
3	95.6	0.06	0.24	49.0					

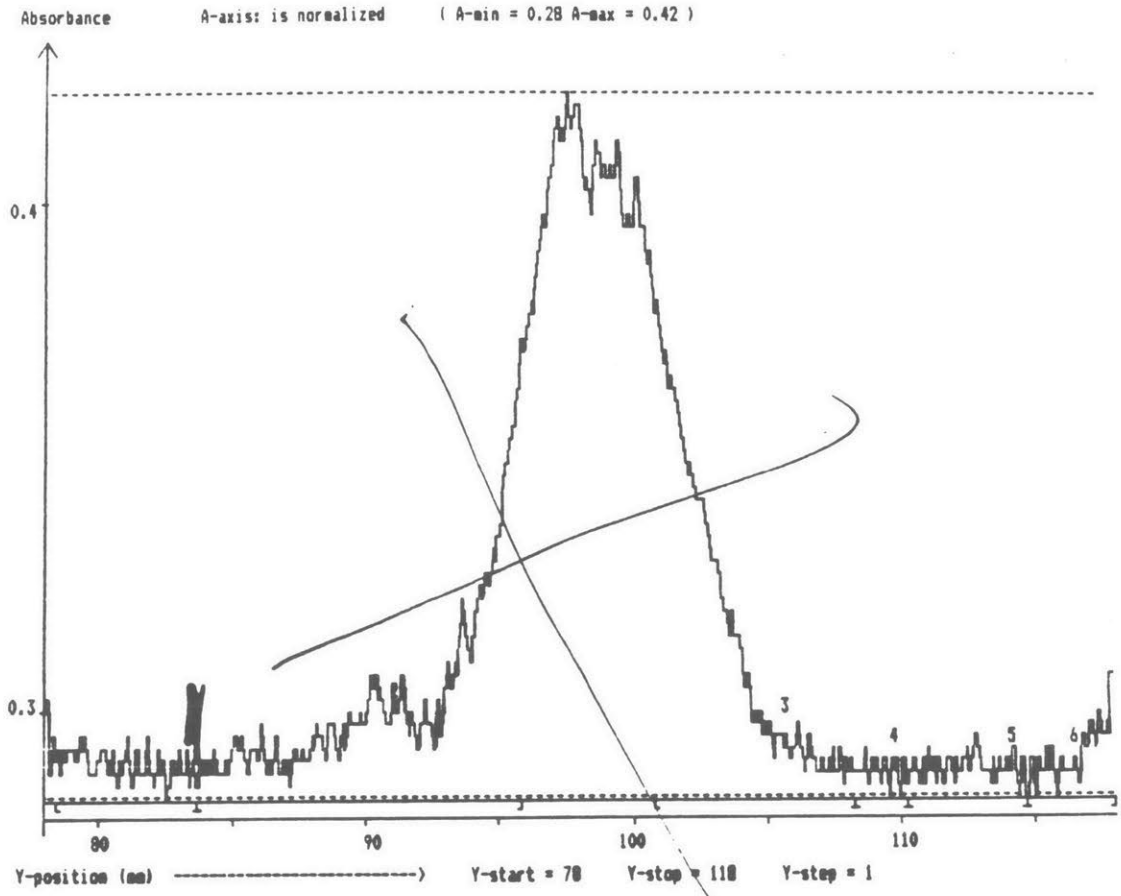
.43

Figure APB: A densitometric scan of a typical Northern blot band. A typical, clean band of a Northen blot was scanned by the LKB Densitometer. The scan is normalized so as to fill the output box. Parameters have been set as detailed in the text. Peak limits are assigned as described in the text. The absorbance of the peak is circled in the table below.



PEAK #	POSITION mm	HEIGHT AU	AREA AU*mm	REL. AREA %	PEAK #	POSITION mm	HEIGHT AU	AREA AU*mm	REL. AREA %
1	104.7	0.01	0.05	3.5	2	109.0	0.50	1.41	96.5

Figure APC: An example of a rare densitometric scan. A scan of a band of a Northern blot in which the peaks were oddly assigned by the densitometer as denoted by in the text. Other parameters have been detailed in the text.



PEAK #	POSITION mm	HEIGHT AU	AREA AU*mm	REL. AREA %	PEAK #	POSITION mm	HEIGHT AU	AREA AU*mm	REL. AREA %
1	83.1	0.00	0.03	5.7	4	109.8	0.01	0.01	1.7
2	91.2	0.02	0.23	40.5	5	114.0	0.00	0.02	4.0
3	105.5	0.01	0.25	43.6	6	116.4	0.00	0.03	4.6

displaying first order kinetics is that of "half-life", the time it takes for one half of the substance to disappear. Half-life ($t_{1/2}$) is defined as:

$$t_{1/2}=.693/k$$

where:

k=2.303 x slope of the best-fit line through the data points of the logorithm$_{10}$ of the concentration of the reactant plotted against time (Morris, 1974).

The following mathematical manipulations were all performed on a MacIntosh personal computer using the spreadsheet program, Excel. The densitometric absorbance reading for the experimental probe (human c-myc for the heat shock promoter, human β-globin for the metallothionein promoter, and human c-fos, mouse c-fos, and human β-globin for the human c-fos promoter) of a given lane was divided by the densitometric absorbance reading of the same lane which had been hybridized with a normalizing probe (rat α-tubulin or mouse LDH). For both these normalizing probes, it was assumed that the treatment used for inducing the promoter did not affect the levels of that message as is true in the case of Swiss 3T3 cells (Thomas and Thomas, 1986). This seemed to hold true in that the normalizing message gave a fair representation of the ethidium bromide stained gel. Normalizing, in this way, accounts for any error in attempting to load equal amounts of cytoplasmic RNA in each lane. For each exposure that was shown to be in the linear range, the logorithm$_{10}$ of the normalized values for the experimental probe was determined. These values were plotted on the y-axis against time on the x-axis. (For example, see Figures FOSS-FOSV.) The highest normalized value was used as the starting concentration of RNA and the lowest normalized value which was higher than

the zero time value (which was undetectable in most instances) was used as the final concentration point. The slope of the best-fit line through these data points was used to calculate the k-value in the above equation.

Statistical Analysis:

Statistical analyses were also performed on a MacIntosh personal computer using the program, Statworks. The slope and standard error of the slope of the best-fit line through the data points were calculated by Statworks. Half-lives and standard error of half-lives were calculated by hand using these slopes and errors of the best fit lines and the above equation. The t-statistic was calculated by dividing the half-life of a given experiment by its standard error. Using this value and the degrees of freedom in the model (1 less than the number of data points), the P-value was determined from a t-value table (the distribution of t) in Snedecor and Cochran, 1967, p.549. If $P<.05$, the model of decay of cytoplasmic RNA with time was considered acceptable. This means that only 5 in 100 times will the P-value suggest that the model of decay with time is correct when it is not. This is a commonly used statistical cutoff (Snedecor and Cochran, 1967). Since the degrees of freedom are used to assess the P-value and, hence, the acceptability of the half-life, it is clear that more scatter is tolerated if more data points exist on the best-fit line. Most experiments that had to be discarded because of an unacceptable P-value had few data points.

Chapter VII

Materials and Methods

Materials and Methods

Cells and Tissue Culture: All cells were grown in DME (Dulbecco's modified Eagles medium) with 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Media for MEL cells was supplemented with 15% fetal calf serum (Armour Biochemicals, Tarrytown, N.Y.). Media for 3T3 cells was supplemented with 10% calf serum for growth (GIBCO Laboratories, Grand Island, N.Y.), 5% platelet poor plasma (see below) for growth factor deprivation for experiments or 15% calf serum for growth factor refeeding for experiments.

Cell Selection: Transfections and protoplast fusions were selected in 0.5 mg/ml active G418 two days after the transfection procedure. Cells were maintained in this concentration of G418 until initial freezing for maintenance of the cell line. Cells were thawed into media with .25 mg/ml G418 and placed in .5 mg/ml G418 once the cells had well recovered. Experiments were all performed in the absence of G418.

Platelet-Poor Plasma: (Generously supplied by A. Kitchen and J. Townes of B.H. Cochran's laboratory.) Human plasma obtained from the American Red Cross was spun at 16,000 r.p.m. for 20 minutes to remove platelets and fat from the top. It was heated to 56^{o}C for 30 minutes and respun at 12,000 r.p.m. for 20 minutes. The supernatant was dialyzed against .15 M NaCl (6,000 M.W. cut off) overnight and respun at 18,000 r.p.m. for 1.5-2 hours. The fat was again aspirated off the top, filter sterilized and stored in aliquots at -20^{o}C.

Dialysis of serum: Serum was dialysed in an attempt at lowering the basal level of metallothionein-promoted message by reducing the concentration of heavy metal ions and steroids in the serum. Fetal calf serum (500 mls) was dialysed in sausage-shape boiled dialysis tubing (Spectrapor #1, molecular weight cut off 6,000 -8,000) against 30 liters, in several changes, of PBS_A. Serum was then filter-sterilized, and stored frozen at -20°C.

Preparation for Cytofluorimetry: At each time point a 60 mm tissue culture plate of cells was trypsinized, the cells were pelleted and resuspended in 1 ml PBS_A. 10 ml of 70% EtOH was added, mixed well and the tube was stored up to several days at 4°C. This was repeated with 40% EtOH, left on ice 30 minutes and the cells were repelleted. They were resuspended in 1 ml of PBS_A containing 300 μg of pancreatic RNase and incubated for 2 hours at 37°C, rotating. Again, the cells were repelleted and resuspended in .5 ml of PBS_A containing 50 μg/ml propidium iodide. After 15 minutes the cells were then ready for cytofluorimetry.

CAT Assay: CAT activity was assessed as in Gorman et al. (1982) with the following exceptions. Cells were washed 3X in PBS without Ca^{++}/Mg^{++} prior to lysis. Plates of cells sat at room temperature with 1 ml of .04 M Tris, pH 7.4/ 1 mM EDTA/.15 M NaCl for 5 minutes, and then scraped with rubber policemen into eppendorf tubes. Cells were pelleted by 2 minutes in an eppendorf at 4°C and then resuspended in 100 μl .25 M Tris, pH 7.8. Cells were lysed by three cycles of freeze-thawing in dry ice/EtOH and 37°C.

Transfection: Plasmid DNA was transfected into 3T3 cells (NIH or Balb/c/ as stated in the text) using the calcium phosphate co-precipitation technique (Rice and Baltimore, 1982). If the plasmid did not carry the

neomycin resistance gene (TN5), it was cotransfected at a molar ratio of 10:1 with PSV2NEO (Figure HSPC) or PMSVNEO (Figure HSPA) as stated in the text. The precipitated or co-precipitated DNA was resuspended in 1 ml HBS (21 mM Hepes/ 137 mM Nacl/ 5 mM KCl/ 5 mM glucose/ .7 mM Na_2HPO_4, pH 7.05-7.15) per 100 mm tissue culture dish to be transfected. 2 M $CaCl_2$ was added slowly, with gentle mixing until a cloudy, bluish precipitate was visible. After 5 minutes at room temperature one of the following two methods were employed.

Method A: Media was removed form 40-70% confluent recipient cells. Cells were washed one time with serum-free media and the precipitate was gently dropped onto the cells. The plates sat for 20 minutes at room temperature at which time 10 mls of media were added to the plates. They were returned to 37°C for 4 hours. At this point the media was removed from the cells and replaced with 2 mls of HBS/glycerol (HBS/ 15% glycerol) for 2 minutes at 37°C. Quickly, the cells were copiously washed with serum-free media and then returned to 37°C with media. Two days later the cells were selected in G418.

Method B: The precipitate was dropped through the media onto the cells and the plates were placed at 37°C for 12-16 hours. At this point the cells were washed well with serum-free media and either split 1/5 if confluent or nearly confluent ($CaCl_2$-DNA precipitate is mitogenic as well as toxic) or placed at 37°C with media. Two days later the cells were selected in G418.

In both methods, mock-transfected controls were carried through the procedure to ensure that the selection was working. In no case were colonies resistant to G418 without having received the gene.

Protoplast Fusion:

Protoplast formation: Protoplasts were made similarly to the procedure described by Sandri-Goldin et al. (1982). A loopful of the desired bacterium was seeded in 10 mls of M9+CAA (M9 salts (Miller, 1972) containing 0.5% Casamino Acids, 0.4% glucose, 0.012% $MgSO_4$, 5 μg/ml thiamine) with the appropriate antibiotic and grown overnight to saturation. One ml of the overnight culture was added to 100 mls of M9+CAA and antibiotic and grown to $O.D._{600}$ = .7-.8. At this point chloramphenicol was added to 200 μg/ml and shaken for 12-16 hours 3500 r.p.m. and treated with extreme care and caution from here on. The bacteria were resuspended in 6 ml of 20% sucrose/.05 M Tris pH 8.0. One ml of lysozyme solution (2.5 mg/ml in .25 M Tris pH 8.0) was added and stored on ice for 5 minutes. Two ml of .25 M EDTA pH 8 was added and stored on ice for 5 minutes. Very gently and very slowly, with constant rotation of the tube, 2 ml of .05 M Tris was added. The tube of cells was placed at 37°C for up to 20 minutes, and monitored intermittantly by phase-contrast microscopy for the formation of round protoplasts from rod-like bacteria. 70-90% round was used. 40 mls of DME/ 10% sucrose/ 12.5 mM $MgCl_2$ was added drop by drop, with constant, gentle swirling and then left at room temperature while the cells were being prepared. The host bacterium was DH-1.

Cell preparation and fusion: In each fusion, 12 wells of 6-well tissue culture dishes were used. Three wells were given bacteria and no PEG. Three wells were given PEG and no bacteria. Six wells were given both bacteria and PEG. $2X10^6$ cells/well were plated. 4.3 ml of protoplast solution was used per well such that there were about 3000 protoplasts per cell. The total number of cells required were spun at 1000 r.p.m. for 10 minutes in 4 tubes and resuspended in either protoplast solution or media. Each tube was split equally into 3 wells and spun onto the plates at 1800

r.p.m. for 10 minutes in the Sorvall RT6000 with the appropriate adapter for dishes. The media was aspirated off carefully and replaced with fresh 45% PEG (in DME). After 60 seconds at room temperature the plates were flooded with serum-free media, forcing the cells off the plates, and transferred to 50 ml tubes keeping appropriate wells separate. The plates were washed further and added to the tubes Cells were spun down at 1000 r.p.m. for 10 minutes. Each tube of cells was resuspended in 10 ml of media with 10 μg/ml tetracycline and transferred to a T25 flask. (Tetracycline was used as long as the gene for resistance to it was not carried on the plasmid.)

Plamid DNA isolation:

From minipreps: As done by Ish-Horowicz and Burke (1981), with some modifications. 1 ml of overnight culture was spun down in an eppendorf centrifuge for 2 minutes at 4^{o}C. The pellet was resuspended in 100 μl .05 M glucose/ .025 M Tris pH 8.0/ .01 M EDTA, and let stand for 5 minutes at room temperature. 200 μl .2 N NaOH/ 1% SDS is added, vortexed and let to sit on ice for 5 minutes. 150 μl 5 M KOAC pH 4.8 and 400 μl phenol:chloroform:isoamyl alcohol::25:24:1 are added vortexed and spun for 5 minutes at 4^{o}C. The supernatant is precipitated with 1 ml ETOH for 20 minutes at -20^{o}C, spun for 15 minutes and the pellet was washed with 70% ETOH. The dried pellet was resuspended in 26 μl $T^{10}E^{1}$ (10 mM Tris/ 5 mM EDTA). 5-7 μl/lane was used of gel for digestion and 1 μl of 1 mg/ml pancreatic RNase (boiled 10 minutes) was added to remove RNA.

From maxipreps: The procedure for minipreps was scaled up. The bacteria were spun down at 7000 r.p.m. for 12 minutes and 25 ml of the sucrose solution was added to the pellet per liter of bacteria. 60 ml of SDS/NaOH and 30 ml of KOAc were added when required. At this point the solution was

spun down and the supernatant was precipitated with 80 ml of isopropanol. After 20 minutes at -20°C the bottles were spun again and the dried pellets were resuspended in 31 ml of $T^{10}E^{1}$. 32 grams of cesium chloride were added and then 1 ml of 10 mg/ml ethidium bromide. The solution was spun at 45,000 r.p.m. for 18-24 hours in a VTi50 rotor. The bottom, larger plasmid band was pulled and respun for 48 hours in a Ty65 rotor. The band was again pulled, isopropanol-extracted several times to remove the ethidium bromide, dialysed against $T{\cdot}5E{\cdot}05$. The DNA was then precipitated and resuspended at 1 $\mu g/\mu l$.

DNA Manipulations: Standard DNA manipulations were carried out as described in Maniatis et al., (1982). Any non-standard applications were detailed in the appropriate figure legend or elsewhere in the materials and methods. Competent bacteria were made by standard $CaCl_2$ treatment or by the method of Hanahan and Meselson (1980). Transformations into bacteria were standard. Manipulations with M13 and were as described in the BRL (Bethesda Research Laboratories, Bethesda, Maryland) manual on M13.

Construction of fos-glo junction in GLO5 and derivative plasmids (Figure FOSH): For the annealing reaction, 20 pmoles phosphorylated mutagenic oligomer, 20 pmoles unphosphorylated universal primer and 1 pmole of single stranded M13MTFOSGLO template were incubated at 65°C for 10 minutes in 20 mM Tris pH 7.5/ 10 mM $MgCl_2$/ 50 mM NaCl/ 1 mM dithiothreitol in a total volume of 10 μl. After 5 minutes at room temperature the tube was placed on ice. An equal volume of extension/ligation mixture (20 mM Tris pH 7.5/ 10 mM $MgCl_2$/ 10 mM dithiothreitol/ 1 mM each dNTP/ 1 mM ATP/ 3 units T4 DNA ligase/ 2.5 units Klenow fragment of DNA polymerase I) was added, mixed and incubated overnight at 15°C. The transformation was into JM101 as standard.

RNA Isolation: Total RNA (for screening cell lines) was isolated by a modification of the method of Chirgwin et al. (1979). Cells were lysed in guanidinium hydrochloride buffer (7.5 M guanidinium hydrochloride/ 25 mM sodium citrate pH 7.0/ .5% sodium lauroyl sarcosine/ 100 mM β-mercaptoethanol), scraped with a rubber policeman into a 15 ml centrifuge tube, sheared with an 18 gauge needle and layered atop a $CsCl_2$ cushion as described. (Sometimes the samples were stored at -80°C for up to a month prior to being run through the gradient.) RNA pellets were resuspended in guanidinium buffer as RNA is very soluble in it, and precipitated with CH_3COOH and EtOH. Precipitates were reprecipitated with NaOAc and EtOH and washed several times with 70% ethanol. Dried pellets were resuspended in H_2O and the quantitation determined by optical density at 260nm.

Cytoplasmic RNA was isolated for experiments as described in Maniatis et al. (1982) with the following modifications. After SDS/Proteinase K treatment, the aqueous phase was extracted 2 times with phenol, 1 time with phenol:chloroform:isoamy alcohol::25:24:1, and 2 times with chloroform:isoamyl alcohol::24:1. The RNA was then precipitated with NaOAc/EtOH overnight. The pellets were resuspended in H_2O, transferred to eppendorf tubes and precipitated again. The pellets were washed with 3M NaOAc and then precipitated one more time. The pellets were washed 2-3 times with 70% EtOH, dried, resuspended in H_2O, freeze-thawed into solution, if necessary, and quantitated as above.

SP6 RNAse Protection Assay: SP6 transcript was body-labeled after linearization. The DNA was put into SP65 from 3' to 5' so that the complement to the sense strand was transcribed. A 50 μl reaction containing 40 mM Tris pH 7.5/ 6 mM $MgCl_2$/ 2 mM spermidine/ 10 mM dithiothreitol/ 50 units RNasin (Promega Biotec)/ 500 μM each rNTP except

50 μM CTP/ 1 μg linearized plasmid/ 100 μg BSA/ 50 μCi [^{32}P]-CTP/ 1 unit SP6 polymerase (NEN, Boston, Mass.) was incubated for 1 hour at 37°C to synthesize the transcript. The reaction was heat killed at 65°C for 10 minutes, treated with DNase (RNase free, Worthington Biochemicals) at 37°C for 10 minutes to remove template, phenol:chloroform extracted 2 times with the addition of 10 μg yeast tRNA, and precipitated 3 times with ammonium acetate and then 1 time with NaOAc to remove unincorporated ribonucleotides. 30 μg of precipitated total RNA was resuspended in 30 μl hybridization buffer containing 40 mM PIPES pH 6.7/ 400 mM NaCl/ 1 mM EDTA/ 1 X 10^5 cpm Cerenkov of [^{32}P]-riboprobe, incubated 5 minutes at 90°C and then overnight at 37°C. 300 gml of RNase digestion mixture containing 10 mM Tris pH 7.5/ 5 mM EDTA/ 300 mM NaCl/ 40 μg/ml RNase A and 2 μg/ml RNase T1 was added and then incubated for 30 minutes at 37°C. The RNase digestion was stopped by the addition of 20 μl of 10% SDS and 50 μg Proteinase K which is incubated at 37°C for 15 minutes. 20 μg carrier tRNA was added and the mixture was extracted with phenol: chloroform: isoamyl alcohol 2 times. After precipitation and washes in 70% EtOH the sample was run on a sequencing gel and autoradiographed.

RNA gel and transfer to Northern Blot: RNA was run in a formaldehyde, denaturing agarose gel system. Running buffer contained 20 mM MOPS (morpholinopropane sulfonic acid) pH 7.0/ 5 mM NaOAc/ 1 mM EDTA. The sample was heated to 55°C for 15 minutes in 2.2 M formaldehyde, 50% formamide and gel running buffer. Afer the addition of dyes the samples were applied to the gel which contained 1.1% agarose, 1x gel buffer, 2.2 M formaldehyde and .5 μg/ml ethidium bromide for visualization. The gel was run overnight at 18-30 volts with circulating buffer. After photographing the gel, it was rinsed in H_2O for 20 minutes and then in 1X transfer buffer

(12 mM Tris pH 7.5/ 6 mM NaOAc/ .3mM EDTA) for 20 minutes. The transfer membrane, Gene Screen Plus (New England Nuclear, Boston, Mass.), was also wet in H_2O and soaked in transfer buffer for 20 minutes. The gel was electrotransferred (Hoefer Scientific Instruments) onto the filter membrane in transfer buffer for 2 hours at 10 volts, .25 amp and further for 2 hours at 40 volts, 1 amp. The membrane was then rinsed in transfer buffer to remove pieces of agarose and baked for 2 hours in vacuo to reverse the formaldehyde reaction.

Hybridization of blots: Baked blots were prehybridized overnight in 1 M NaCl, 10% dextran sulfate and 1% SDS in a rotating H2O bath at 63°C or 72°C for the human c-fos probe. For hybridization the prehybridization mixture was removed and to the bag was added fresh mixture described above with 100 μg/ml salmon sperm DNA and probe which had been boiled together for 10 minutes. Hybridization was performed for 18-24 hours as described for the prehybridization.

Labeling of probe: DNA was labeled using the OLB (Oligo Labelling Buffer) as described by Feinberg and Vogelstein (1983) with the following modification. DNA was not isolated from the gel, but was run in a lowmelt agarose gel. The reaction was performed in the presence of agarose. All probes were run in agarose except for the .39 kb human c-fos DNA which was isolated from the gel.

Washing of blots: Blots were washed as follows: (20X SSCPE= 3 M NaCl/ .3 M NaCitrate/ .3 M KH_2PO4/ 20 mM EDTA) 2X at room temperature for 20 minutes each in 2X SSCPE, 1% SDS, then 2X at 55°C for 30 minutes each in 2X SSCPE, 1% SDS, then 2X at 55°C for 30 minutes each in .5X SSCPE, 1% SDS, then 2X

at 55°C for 30 minutes each in .25X SSCPE, .5% SDS. Blots hybridized with human c-fos DNA were further washed 2X at 60°C for 30 minutes each in .1X SSCPE, .5% SDS. Blots were then rinsed in 1X SSCPE briefly and exposed to film wrapped in Saran Wrap (Dow) as described in the Appendix.

Slot Blot Procedure: RNA was treated with 3 volumes of 6.15 M formaldehyde, 10X SSCPE at 65°C for 15 minutes. The samples were then shifted to ice and loaded onto Gene Screen Plus (NEN) filter via Schliecher and Schuell Slot Blot II using the accompanying literature. The membrane was baked for 2 hour in vacuo to reverse the formaldehyde reaction.

Polysome gradients: Cytoplasmic extracts from 3 confluent 150 mm tissue culture plates were prepared as for the isolation of cytoplasmic RNA, except that RNAsin was used in lieu of vanadyl-ribonucleoside complexes. Extracts were either frozen in liquid nitrogen for later use or made to .5 M KCl and layered atop a 10-50% sucrose gradient containing .5 M KCl/ 5 mM MgCl2/ 20 mM Hepes, pH 7.2/ 2 mM dithiothreitol/ 1 mg/ml heparin sulfate. Gradients were centrifuged for 4 hours at 25,000 r.p.m. at 4°C in an SW 27 rotor. Gradients were analyzed in an ISCO UA5 absorbance monitor and an LKB gradient fraction collector (generously made available by H. Munro). Collected fractions were precipitated overnight with two volumes of EtOH at -80°C. Fractions were pooled into 3 sections: mRNPs and 40S subunit, 60S subunit plus monosomes and disomes, and polysomes using 70% EtOH, and then washed copiously with 70% EtOH to dissolve the sucrose. The pellets were then resuspended in H_2O and treated as samples for the extraction of cytoplasmic RNA starting with SDS/Proteinase K treatment.

Trypan Blue exclusion: Cells were mixed 1:1 with .5% trypan blue dye in PBS_A. Mixture was monitored in a hemocytometer in which viable cells successfully excluded the dye.

Chapter VIII

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