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Translational efficiency of polycistronic mRNAs and their utilization to express heterologous genes in mammalian cells

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The translation of polycistronic mRNAs in mammalian cells was studied. Transcription units, constructed to contain one, two or three open reading frames (ORFs), were introduced stably into Chinese hamster ovary cells and transiently into COS monkey cells. The analysis of mRNA levels and protein synthesis in these cells demonstrated that the mRNAs transcribed were translated to generate multiple proteins. The efficiency of translation was reduced ~40- to 300-fold by the insertion of an upstream ORF. The results support a modified 'scanning' model for translation initiation which allows for translation initiation at internal AUG codons. High-level expression of human granulocyte–macrophage colony stimulating factor was achieved utilizing a vector that contains a polycistronic transcription unit encoding an amplifiable dihydrofolate reductase marker gene in its 3′ end. Thus, polycistronic expression vectors can be exploited to obtain high-level expression of foreign genes in mammalian cells.

Key words: polycistronic transcription unit/gene expression/gene transfer/expression vectors

Introduction

Although the precise mechanism by which eukaryotic ribosomes recognize particular initiation sites in mRNA molecules is unclear, a substantial amount of evidence supports a 'scanning' model for translation initiation (Kozak, 1980a). This model suggests that a 40S ribosomal subunit binds to the 5′ capped end of the mRNA and migrates in the 3′ direction until it encounters the first AUG triplet which, if present in an appropriate context, can efficiently serve as the initiator codon. The scanning model is supported by the stimulatory effect of the m^{7}G cap (Shatkin, 1976), the inability of ribosomes to bind circular mRNAs (Kozak, 1979a; Konarska *et al.*, 1981), the ability of 40S subunits to migrate along the mRNA (Kozak and Shatkin, 1978; Kozak, 1979b, 1980b), and the negative influence of significant secondary structure and of additional AUGs inserted between the 5′ end of the mRNA and the initiation codon (Kozak, 1984, 1986; Liu *et al.*, 1984; Pelletier and Sonnenberg, 1985). The finding that eukaryotic ribosomes usually translate only from the 5′ proximal AUG in polycistronic mRNAs is also supportive of a scanning model. However, many naturally occurring mRNAs are known to initiate at internal AUGs (Ghosh *et al.*, 1978; Wengler *et al.*, 1979; Perler *et al.*, 1980; Bos *et al.*, 1981; Hagenbuchle *et al.*, 1981; Hendy *et al.*, 1981; Vogeli *et al.*, 1981; Clerx-van Haaster *et al.*, 1982; Swanstrom *et al.*, 1982; Yoo *et al.*, 1982; Marsden *et al.*, 1983; Hughes *et al.*, 1984; Dixon and Hohn, 1984). In addition, several studies have demonstrated translation initiation from internal AUG codons in experimental constructs, by the insertion or deletion of initiator or terminator codons upstream from the translation start site of a particular open reading frame (ORF) (Dixon and Hohn, 1984; Hughes *et al.*, 1983; Kozak, 1984; Liu *et al.*, 1984). These studies demonstrate that the insertion of an upstream AUG, which is out of frame with the downstream ORF, can severely suppress translation of the downstream ORF. Insertion of a termination codon can reverse the suppression. Thus, these studies suggest a modified scanning model with the potential for reinitiation at internal, downstream AUGs. However, to date there have been few reports that polycistronic mRNAs can actually translate two or more proteins from non-overlapping ORFs in the same transcription unit in mammalian cells (Mertz *et al.*, 1983; Herman, 1986; Peabody *et al.*, 1986; Peabody and Berg, 1986).

We have examined the translational efficiency of polycistronic mRNAs in mammalian cells. Plasmids harboring transcription units constructed to contain one, two or three ORFs encoding protein products that can be easily monitored, were introduced into Chinese hamster ovary (CHO) cells by stable transformation or into COS monkey cells by transient transfection. The results demonstrate that both cell types are capable of translating multiple non-overlapping ORFs from a single polycistronic transcript. However, the efficiency of translation from an ORF was dramatically reduced by the presence of upstream ORFs. Direct selection for the gene product encoded from the 3′ proximal ORF is demonstrated to provide a useful means to obtain expression of heterologous genes in mammalian cells.

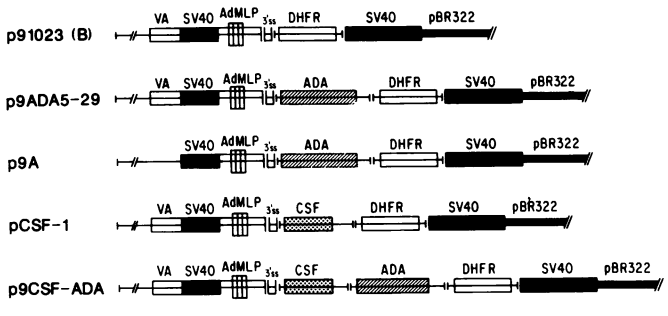

Fig. 1. Structure of polycistronic expression vectors. The derivation of these vectors is described in Materials and methods. The functional elements of the expression vectors are depicted and have been described previously (Kaufman and Sharp, 1982a,b). All vectors utilize the adenovirus major late promoter for transcription initiation and contain the majority of the adenovirus tripartite leader present on adenovirus late mRNAs. A small intron is present consisting of a 5′ splice site from the adenovirus first late leader and an introduced 3′ splice site. In addition the vectors contain the SV40 origin of replication and transcriptional enhancer, and the SV40 early polyadenylation signal. Four of the vectors contain the adenovirus VA genes (VA) as shown. All vectors are contained in a derivative of pSVOd (Mellon *et al.*, 1981). The murine DHFR, murine ADA and human GM-CSF (CSF) coding regions are represented. The 3′ untranslated region of GM-CSF is 330 bases and contains an ORF of 273 bases. The 3′ untranslated region of ADA is 147 bases and contains a short ORF of 18 bases. The 5′ untranslated region of DHFR is 105 bases, contains no AUGs and has an 18-bp poly(G) tail from the cDNA cloning (Kaufman and Sharp, 1982b). The 5′ untranslated region of ADA contains an *Eco*RI site (GAATTCATG) at the translation initiation site.

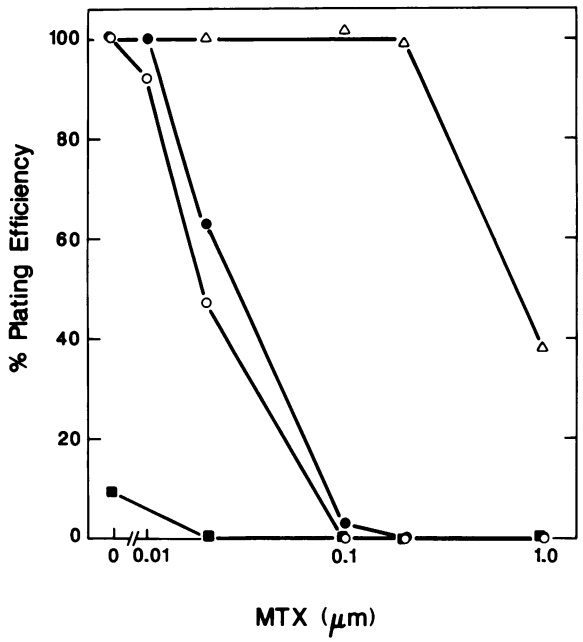

Fig. 2. Plating efficiency versus MTX concentration of ADA transformants before and after ADA amplification. The plating efficiency for an original ADA transformant (1−0.1, ■) grown in 0.1 μM 2′-deoxycoformycin and two amplified transformants (1−100, ○; and 2−100, ●) grown in 100 μM 2′-deoxycoformycin were determined by plating 200 and 2000 cells per 10-cm dish in nucleoside-free alpha media with increasing concentrations of MTX. Colonies were stained and counted 12 days after plating. Values are expressed as a per cent of the control (media containing 1.1 mM adenosine, 1 mM uridine, 10 μg/ml deoxyadenosine, 110 μg/ml thymidine, and without 2′-deoxycoformycin and MTX) which was 23% for the clone 1−0.1, 30% for the clone 2−0.1 and 22% for the clone 1−100. Also shown is the plating efficiency of clone E6−0.1, which is a cell line selected for growth in 0.1 μM MTX as a result of expression from p91023(B) (△).

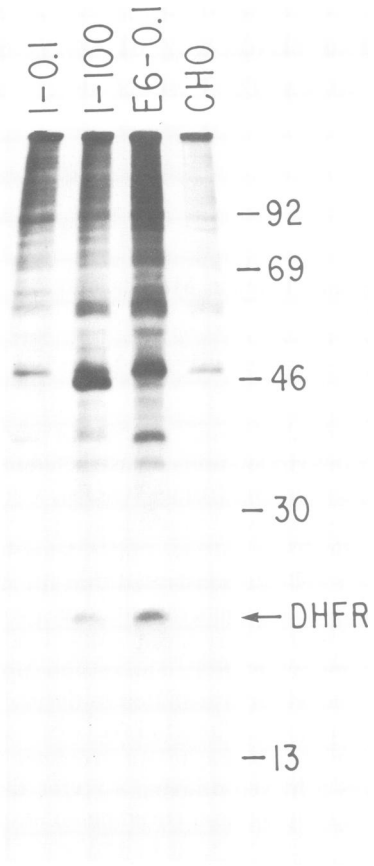

Fig. 3. Analysis of DHFR synthesis in ADA amplified and control CHO cells. Cells were labeled for 45 min with [^{35}S]methionine and cell extracts prepared for immunoprecipitation as described by Kaufman *et al.* (1985) with a rabbit anti-DHFR antibody. The cell lines indicated are clone 1 in 0.1 μM (1−0.1) and 100 μM (1−100) 2′-deoxycoformycin, another CHO cell line resistant to 0.1 μM MTX after transfection with p91023(B) (E6−0.1), and the original CHO cells (CHO). The band migrating at 45-kd in the 1−100 cells is ADA.

Results

Translation of a dicistronic mRNA in stable transformed CHO cells

The potential of polycistronic mRNAs to generate multiple proteins from non-overlapping reading frames was studied by the construction of vectors to transcribe mRNAs encoding multiple ORFS (depicted in Figure 1), and analysis of the proteins expressed after their transient introduction into COS monkey cells, or stable introduction into CHO cells. p9ADA5-29 (Figure 1), which encodes a dicistronic adenosine deaminase−dihydrofolate reductase (ADA−DHFR) transcription unit, was introduced into DHFR-deficient CHO cells, and transformants isolated by selection for ADA expression (Kaufman *et al.*, 1986a). Two transformants were randomly chosen and propagated in increasing concentrations of 2′-deoxycoformycin, a tight-binding inhibitor of ADA. This selection resulted in cells which were resistant to 100 μM 2′-deoxycoformycin, and which contained ~500 copies of the plasmid DNA integrated into the CHO cell genome (Kaufman *et al.*, 1986a). These cells, which were initially DHFR-deficient, were then analysed for their level of DHFR expression. In order to monitor functional DHFR expression from the dicistronic transcription unit, the cells were grown under conditions that require functional DHFR, i.e. growth in the absence of added nucleosides. In addition, the growth potential as a function of increasing concentrations of methotrexate (MTX) was determined to provide an indication of the level of DHFR expression. Figure 2 shows the results of a plating efficiency experiment, where an original ADA transformant and two highly amplified clones were propagated in increasing concentrations of MTX. The result demonstrates that 8% of the population of the original transformant grew in the DHFR-selective media. The plating efficiency, in the DHFR-selective media, of the highly amplified cell line was 100%. As the MTX concentration was increased, the plating efficiency decreased, which is consistent with the growth observed being due to function DHFR expression. Low levels of MTX had little effect on the plating efficiency, whereas 0.2 μM reduced the plating efficiency to <1%. In contrast, a cell line selected for resistance to 0.1 μM MTX, by DHFR expression from a monocistronic transcription unit, was capable of growth in MTX concentrations up to 1.0 μM. These results demonstrate a low, but significant, level of DHFR expression from the ADA−DHFR dicistronic expression vector, in the absence of any direct selection for DHFR expression. Thus, it can be concluded that cells which have amplified the ADA−DHFR dicistronic transcription unit appear to express both ADA and DHFR proteins on the basis of the observed resistance of these cells to the appropriate selective agents.

DHFR protein synthesis was directly demonstrated by L[^{35}S]methionine labeling of CHO cells transfected with p9ADA5-29, and immunoprecipitation with a rabbit anti-mouse DHFR antibody (Figure 3). A 20-kd band was found to co-migrate with authentic murine DHFR (Figure 3, cell line 1−100).

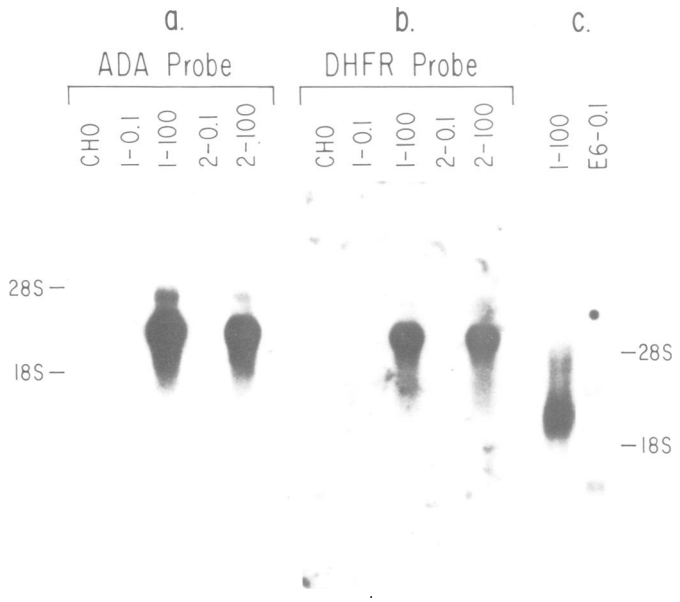

Fig. 4. RNA blot hybridization analysis of original and ADA amplified cell lines. Total RNA was isolated and electrophoresed on formaldehyde–formamide denaturing 1% agarose gels and transferred to nitrocellulose paper as described in Materials and methods. Hybridization as to a nick-translated probe from a fragment of the ADA coding region **(a)** or to the DHFR coding region **(b,c)**. The filter represented in **panel a** was washed and rehybridized with the DHFR probe **(b)**. The cell lines indicated are as described in Figure 2. Indicated are the 18S and 28S rRNA marker bands. The source or the band migrating below 28S in clone E6–0.1 is not known, but it does not appear in poly(A)-containing RNA.

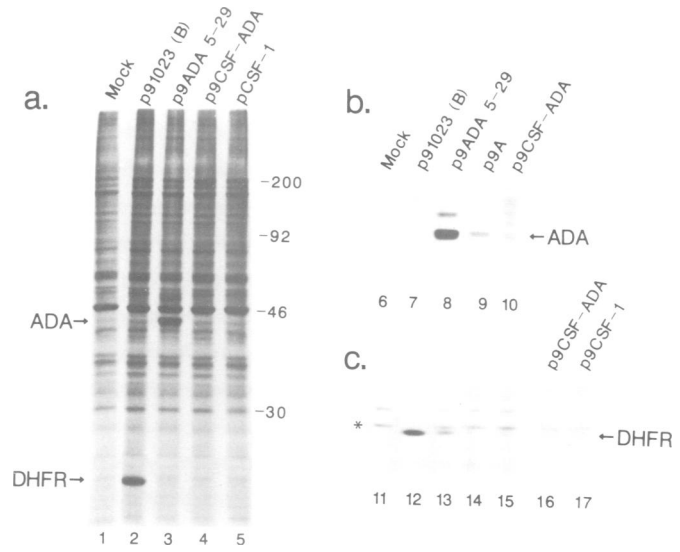

Fig. 5. Analysis of total and immunoprecipitated ADA and DHFR proteins synthesized in transfected COS cells. COS cells were transfected with the plasmids indicated as described in Materials and methods. Sixty hours post-transfection, the cells were labeled with [^{35}S]methionine for 1 h and cell extracts taken for anlaysis by SDS–reducing polyacrylamide gel electrophoresis before and after immunoprecipitation with either an anti-ADA antibody **(panel b)** or an anti-DHFR antibody **(panel c)**. **Panel a** represents the total protein synthesis. Samples 6–10 correspond to samples 11–15, respectively. Samples 16 and 17 were obtained from a separate transfection. The lanes are labeled with the plasmid DNA which was transfected into the cells. Mock refers to cells which did not receive DNA. The bands migrating at 20 and 44 kd are DHFR and ADA as indicated. The band migrating above ADA in **lane 8** is also ADA. The band migrating slightly above the murine DHFR in **panel c** is the monkey DHFR. In order to guarantee quantitative immunoprecipitation, only 1/20 of the cell extract was immunoprecipitated for samples in **lanes 8, 9** and **12** compared with the other lanes. The conditions for the immunoprecipitation were determined by demonstrating that reprecipitation of immunoprecipitated supernatants did not bring down significantly more antigen. Samples 11, 13, 14 and 15 all exhibit similar amounts of endogenous monkey DHFR (*), again demonstrating quantitative immunoprecipitation.

The amount of this protein increased as the level of resistance to 2′-deoxycoformycin was increased: compare cell line 1–0.1, which was resistant to 0.1 μM with cell line 1–100, resistant to 100 μM. This increase is presumably due to amplification of the ADA– DHFR transcription unit in these cells (Kaufman *et al.*, 1986a). For reference, see the level of DHFR produced by the cell line E6–0.1, which has a monocistronic DHFR transcription unit, synthesizes several fold greater levels of DHFR, and is resistant to 0.1 μM MTX compared with line 1–100.

In order to confirm that the DHFR expression osberved was derived from the ADA–DHFR dicistronic mRNA, the cells were examined by DNA and RNA blot hybridization analysis. RNA blot hybridization analysis demonstrated the appearance of a single mRNA, using either ADA- or DHFR-specific hybridization probes, which had the size expected for the mRNA encoded by the dicistronic transcription unit (Figure 4). Comparison with the CHO cell line resistant to 0.1 μM MTX (E6–0.1 in Figure 3) as a result of expression from a monocistronic transcription unit, demonstrated the appearance of a 12-fold less abundant DHFR mRNA in clone E6–0.1 (Figure 4, panel c). Thus, DHFR expression at this level would generate a detectable transcript by this analysis. Comparison of the band intensities in order to quantitate DHFR protein levels in these lines indicated that DHFR synthesis in E6–0.1 is 3-fold greater than in 1–100. Comparison of the abundance of DHFR mRNA species in these two cell lines and of the relative amount of DHFR synthesis (Figures 3 and 4, panel c) indicates that the monocistronic mRNA translates DHFR ~36-fold more efficiently than the dicistronic mRNA derived from p9ADA5-29.

Southern blot analysis by hybridization to a probe specific to the 5′ end of the DHFR coding sequence indicated a single DHFR sequence had integrated and that no rearrangement of sequences surrounding the integrated DHFR DNA had occurred as a result of selection for ADA gene amplification (data not shown).

Translation of dicistronic and tricistronic mRNAs in transiently transfected COS monkey cells

The results from CHO cells indicate that dicistronic mRNAs can be translated, but the translation efficiency of the downstream ORF, in the construct tested, is dramatically reduced. In order to determine if the introduction of another ORF, upstream of DHFR, has further suppressive effects, an ORF encoding human granulocyte–macrophage colony stimulating factor (GM-CSF) was introduced 5′ of the ADA ORF to obtain p9CSF-ADA (Figure 1). In order to assay more conveniently for translation of the resultant polycistronic mRNAs, these plasmid DNAs were transiently introduced into COS monkey cells. Seventy-two hours post-transfection, the cells were labeled for 1 h with L[^{35}S]methionine and cell extracts prepared for immunoprecipitation and SDS–polyacrylamide gel electrophoresis (Figure 5). Cells transfected with p9CSF-ADA express ~1 μg GM-CSF/10^6 cells/day. Results demonstrate that DHFR and ADA represent a major proportion (~5%) of the total protein synthesis in cells transfected with p91023(b) and p9ADA5-29 respectively (Figure 5, panel a). DHFR synthesis could not be detected in cells transfected with p9ADA5-29 and p9CSF-ADA by analysis of total protein

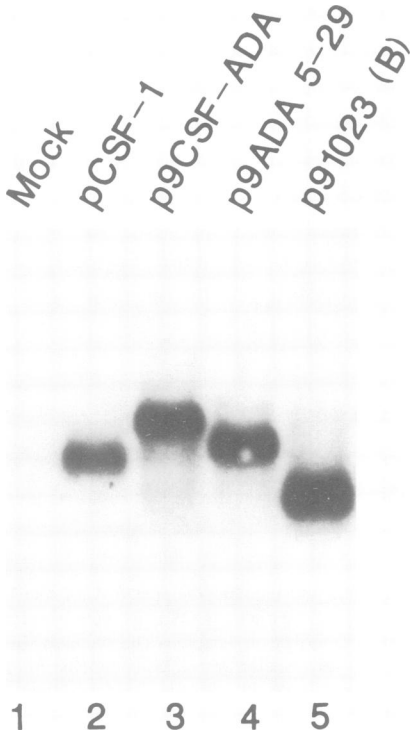

Fig. 6. RNA blot analysis of transfected COS cells. COS cells were transfected with the indicated plasmid as described in Materials and methods. Total RNA was harvested 60 h after transfection and electrophoresed and analysed by RNA blotting procedures as described in Materials and methods. Filters were hybridized to a DHFR cDNA probe prepared by nick-translation of a *Pst*I fragment isolated by gel electrophoresis. The expected sizes of the p91023(B), p9ADA5-29, p9CSF-ADA and pCSF-1 transcripts are 1150, 2290, 3060 and 1920 bases, respectively.

Table I. Summary of COS cell expression of ADA and DHFR from polycistronic transcripts

ORF order (plasmid)	Relative expression level	
	DHFR	ADA
DHFR [p91023(B)]	1	
CSF-DHFR (pCSF-1)	1/300	
ADA−DHFR (p9ADA5-29)	1/100	1
CSF-ADA−DHFR (p9CSF-ADA)	1/300	1/150

Relative levels of ADA and DHFR expression are indicated as quantitated from results shown in Figure 5 as described in Materials and methods.

synthesis on SDS−polyacrylamide gels (Figure 5, panel a). Analysis by immunoprecipitation with anti-DHFR and anti-ADA antibodies is shown in Figure 5 (panels b and c). In order to guarantee quantitative immunoprecipitation, lanes 8, 9 and 12 represent 1/20 of the cell extracts that were immunoprecipitated in the other lanes. Introduction of the ADA ORF, 5′ proximal to DHFR, resulted in a 100-fold decrease in translation of DHFR. Similarly, the introduction of CSF ORF to generate pCSF-1 resulted in a 300-fold decrease in DHFR translation. The introduction of the CSF ORF into p9ADA5-29 to generate p9CSF-ADA results in a 150-fold reduction in ADA translation (panel b, lane 10) when compared with the synthesis of ADA in p9ADA5-29 (panel b, lane 8) but only a 3-fold reduction in DHFR translation relative to p9ADA5-29 (panel c, lanes 13 and 15). Similarly, the introduction of the ADA ORF in between the GM-CSF and DHFR ORFs in pCSF-1 to generate p9CSF-ADA does not result in a decrease in DHFR translation (panel c, lanes 16 and 17). RNA blot analysis demonstrated that the plasmids direct the synthesis of equivalent amounts of single mRNAs of the expected size (Figure 6). Further hybridization analysis with ADA and GM-CSF labeled probes demonstrated that the observed mRNAs contain the expected ORFs (data not shown).

The results from these COS cell experiments are summarized in Table I. The results demonstrate the ability of cells to translate polycistronic mRNAs encoding GM-CSF, ADA and DHFR. However, there is a 100-fold reduction in DHFR translation, by insertion of an upstream ADA ORF, and only a further 3- to 4-fold reduction by the additional insertion of a GM-CSF ORF, 5′ of the DHFR ORF. Thus, the insertion of a single upstream ORF severely suppresses DHFR translation in COS cells, as was found in CHO cells. However, the insertion of an additional ORF has a smaller effect on DHFR translation.

Previous experiments have demonstrated the ability of adenovirus virus-associated (VA) RNA to potentiate translation in transiently transfected COS cells (Kaufman, 1985). The ability of VA RNA to potentiate the translation of polycistronic mRNAs is shown in Figure 5. Expression of DHFR from an ADA−DHFR dicistronic transcription unit contained in p9A (Figure 1) which has deleted the adenovirus VA genes is reduced by 10-fold (Figure 5, panel c, lanes 13 and 14), similar to the 8-fold reduction seen for ADA (Figure 5, panel b, lanes 8 and 9). The differences in ADA and DHFR expression in response to adenovirus VA RNA result from differences in translation, since the level of the dicistronic mRNA is not affected by the presence of VA RNA (data not shown). This result demonstrates that VA affects translation initiation at internal AUGs, in the same way as AUGs at the 5′ proximal end of the mRNA.

Utilization of polycistronic transcription units to efficiently express heterologous genes

The inefficient translation of ORFs present in the 3′ end of mRNAs can provide a unique approach to obtain high-level expression of heterologous genes in mammalian cells, by direct selection for expression of an inefficiently translated, amplifiable marker gene carried in the 3′ end of a particular transcription unit. This has been demonstrated by transformation of DHFR-deficient CHO cells with a plasmid which is capable of encoding GM-CSF and DHFR in a dicistronic transcription unit. The plasmid pCSF-1 was introduced into CHO DHFR-deficient cells by protoplast fusion and transformants selected directly for DHFR expression. A pool of DHFR-positive colonies was propagated in sequentially increasing concentrations of MTX. Analysis of GM-CSF activity indicated that as cells became increasingly resistant to MTX, the level of GM-CSF expression correspondingly increased. The comparison with cells derived by co-transfection of pCSF-1 with DHFR expression plasmids (as described by Kaufman *et al.*, 1985) indicated that selection for the polycistronic GM-CSF−DHFR alone yielded initial transformants which express ~10-fold higher levels of GM-CSF (data not shown).

Analysis of the level of CSF protein synthesis and secretion is shown in Figure 7. Cells selected for resistance to 2.0 μM and 100 μM MTX were labeled with [^{35}S]methionine for 1 h and conditioned media and cell extracts prepared and analysed by SDS−polyacrylamide gel electrophoresis. In comparison with the secreted, labeled proteins from the original DHFR-deficient CHO cells, a smear from 15 to 32 kd is observed in cells (clone D2) resistant to 2.0 μM MTX. These species are specifically immunoprecipitated with a rabbit anti-human GM-CSF antisera. The heterogeneity in mol. wt results from extensive glycosylation

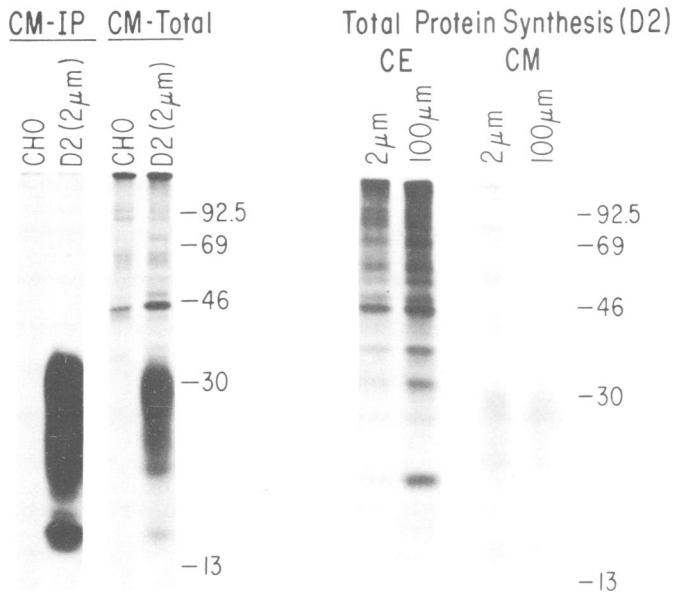

Fig. 7. Synthesis and secretion of human GM-CSF expressed in CHO cells. The GM-CSF producing CHO cell clone D2 resistant to 2 μM and 100 μM MTX was labeled with [^{35}S]methionine for 1 h and cell extracts (CE) and conditioned media (CM) taken for analysis by SDS−polyacrylamide gel electrophoresis before **(middle** and **right panels)** and after **(left panel)** immunoprecipitation with a rabbit anti-human GM-CSF antisera. Also shown is a control of the original CHO cells (CHO).

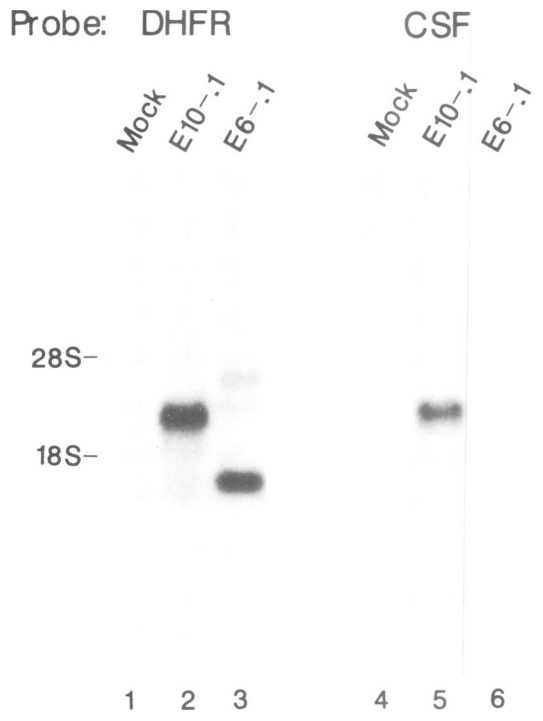

Fig. 8. RNA blot analysis of GM-CSF and DHFR mRNAs in GM-CSF producing CHO cells. Total RNA was isolated from the original CHO cells (mock), the GM-CSF producing pool E10 resistant to 0.1 μM MTX (E10−.1), and another CHO line resistant to 0.1 μM MTX by expression of a monocistronic DHFR transcript (E6−.1) and analysed by gel electrophoresis and RNA blotting procedures. Parallel RNA blots were hybridized to a DHFR probe or a GM-CSF probe prepared by nick-translation of isolated gel fragments.

of CSF and is similar to that observed from activated T cells (Wong *et al.*, 1985). GM-CSF represents the majority of protein secreted in the 2.0 μM MTX-resistant cells and >90% of the total protein secreted in the cells selected for resistance to 100 μM MTX. Upon selection for resistance to higher MTX, there is no apparent change in the nature or extent of glycosylation as monitored by this analysis. The band migrating at 20 kd in the cell extracts is DHFR.

Analysis of the mRNA and the DNA in the CHO cells transformed to the DHFR-positive phenotype with pCSF-1 and subsequently amplified by MTX resistance selection, demonstrated CSF and DHFR expression resulted from a dicistronic mRNA. Northern blot analysis indicated the presence of a single 1.8-kb mRNA, the expected size for the transcript (Figure 8). Southern blot analysis demonstrated a unique band which co-migrated the appropriate *Bam*HI fragment from pCSF-I (data not shown). Since *Bam*HI sites flank the polycistronic transcription unit, the integrity of this fragment was not lost upon transfection and selection for amplification. The copy number in cells resistant to 2 μM MTX is ~2000 copies/cell. By comparison with previous reports (Lau *et al.*, 1984; Kaufman *et al.*, 1985, 1986b), this represents a very high degree of gene amplification at a relatively moderate level of MTX resistance. This high degree of amplification is probably a result of the inefficient expression of DHFR from the dicistronic transcript. The D2 cells resistant to 2 μM MTX contain one or two significant chromosomal homogeneously staining regions (HSRs) and the GM-CSF expression level has been maintained stably for over 1 year in culture in the presence of MTX (data not shown). These are characteristics typical of CHO cells containing high degrees of gene amplification (Kaufman *et al.*, 1983; Lau *et al.*, 1984). These results demonstrate that polycistronic transcription units can provide a convenient means to select cells which efficiently express heterologous genes.

Discussion

Transcription units have been constructed which contain multiple ORFs encoding DHFR, ADA and GM-CSF. The expression of these proteins from a single mRNA has been studied in stably transformed CHO cells and in transiently transfected COS monkey cells. Using a series of polycistronic expression vectors, the translational efficiency was shown to be dramatically reduced by insertion of upstream ORFs. The efficiency of DHFR translation decreased ~40- and 100-fold in CHO and COS cells, respectively, by the insertion of an upstream ADA ORF. Similarly, the 5′ insertion of a GM-CSF ORF, resulted in a 150- and a 300-fold decrease in ADA and DHFR translation, respectively, in the resultant dicistronic mRNAs. The 5′ insertion of the GM-CSF ORF into an ADA−DHFR dicistronic transcription unit resulted in a 150-fold decrease in ADA translation, but only a 3-fold decrease in DHFR translation. The dramatic decrease in DHFR translation by insertion of a single 5′ ORF is in contrast to the small decreases in DHFR translation observed from polycistronic mRNAs by Peabody *et al.* (1986). This may suggest that the primary nucleotide sequences, its length, or mRNA secondary structure may dramatically affect the frequency of internal translation initiation. The negative influence of upstream ORFs on translation initiation is consistent with previous work, which has shown that AUG codons, inserted upstream of the 'correct' AUG initiation codon, reduces translation at the 'correct' site (Dixon and Hohn, 1984; Hughes *et al.*, 1984; Kozak, 1984; Liu *et al.*, 1984). These results suggest that internal initiation occurs by translation reinitiation as opposed to independent in-

ternal initiation. However, results reported here demonstrate 5′ insertion of an ORF to generate a tricistronic mRNA dramatically reduces translation of the second ORF (150-fold) whereas translation of the third ORF is only slightly reduced (3-fold). A possible explanation for this finding is the occurrence of independent internal initiation at internal AUGs. However, our data cannot rule out other possibilities.

Previous studies have demonstrated inefficient translation of some mRNAs after transient transfection of plasmid DNA into cells (Svensson and Akusjarvi, 1984, 1985; Kaufman, 1985). The inefficiency in translation of these mRNAs can be corrected by the presence of adenovirus VA RNA. Although the role of VA RNA in potentiating translation in transfected cells has not been elucidated, its role has been intensively studied in adenovirus-infected cells (Reichel *et al.*, 1985; Kitajewski *et al.*, 1986; O'Malley *et al.*, 1986). These studies have demonstrated that VA RNA is likely to act by increasing the levels of functional eIF-2 by preventing phosphorylation of its alpha subunit through direct inhibition of the double-strand RNA-activated protein kinase (DAI kinase). Studies presented here have demonstrated the ability of VA equally to potentiate translation at internal and 5′ proximal AUGs. These results suggest that internal initiation, similar to initiation at the 5′ end of the mRNA, may be affected by altered levels of eIF-2.

DHFR has been useful as an amplifiable marker and has been exploited to obtain high-level expression of heterologous genes in mammalian cells (Haynes and Weissman, 1983; Scahill *et al.*, 1983; Lau *et al.*, 1984; McCormick *et al.*, 1984; Kaufman *et al.*, 1985, 1986b). However, co-transfection and co-amplification of a heterologous gene with a DHFR gene frequently results in deletion of the desired heterologous gene (Kaufman and Sharp, 1982a; Kaufman *et al.*, 1985). The use of polycistronic expression vectors, that encode transcripts containing the desired coding region upstream of the DHFR coding region, can provide an approach to solve this problem; the heterologous gene and the DHFR gene can be linked on the same transcription unit, and as a result the possibility of deletion may be reduced. The ability to select directly for DHFR expression from a GM-CSF and DHFR polycistronic transcription unit was demonstrated. Selection for cells which are moderately resistant to MTX resulted in cells which have a high level of GM-CSF−DHFR transcript, encoded by a highly amplified transcription unit.

The DHFR co-amplification approach to heterologous gene expression has generally been limited to CHO cells which are deficient in DHFR. The dicistronic ADA−DHFR vector described here provides a unique opportunity to introduce and amplify foreign genes to very high copy number in a variety of mammalian cells. First, since ADA can function as a dominant selectable and amplifiable marker for gene transfer in a variety of cells (Kaufman *et al.*, 1986a), initial transformants can be selected for ADA expression, and the DNA amplified by growth in increasing concentrations of 2′-deoxycoformycin. Second, cells selected to contain a high degree of amplification of the ADA−DHFR polycistronic transcription unit, should also produce a sufficient amount of DHFR to allow MTX resistance selection in order to amplify further the gene copy number. The potential usefulness of this doubly amplifiable vector is currently being evaluated.

Materials and methods

Vector construction

Figure 1 depicts the functional components of the expression plasmids used in this study. The construction of the murine adenosine deaminase (ADA) expression vector p9ADA5-29 has been described by Yeung *et al.* (1985). This plasmid contains the mouse DA cDNA sequence inserted into the *Eco*RI site of p91023(B) (Wong *et al.*, 1985). This plasmid encodes a dicistronic ADA and mouse DHFR transcript. A derivative of p9ADA5-29 lacking the adenovirus VA RNA genes was constructed by digestion of p9ADA5-29 and pL58 (Kaufman, 1985) with *Bgl*II (present in the intron for the transcription unit) and *Sal*I (present in the tetracycline resistance gene), ligation of the large, ADA containing, fragment of p9ADA5-29 to the small, SV40 origin-containing fragment of pL58, and transformation of *Escherichia coli* to tetracycline resistance. The resultant plasmid p9A is identical to p9ADA5-29 except for the deletion of the adenovirus VA genes. The GM-CSF expression plasmid (pCSF-1) (Wong *et al.*, 1985) encodes a dicistronic CSF and DHFR transcript. In order to obtain a tricistronic expression plasmid, the CSF coding region (770 bp) was obtained from pCSF-1 by *Eco*RI digestion and inserted into the *Eco*RI site (5′ of the ADA sequence) in p9ADA5-29 to create p9CSF-ADA.

Cell culture

COS monkey cells were transfected using DEAE−dextran with the addition of a chloroquin treatment as described (Sompayrac and Dana, 1981; Luthman and Magnusson, 1983; Kaufman, 1985).

DHFR-deficient CHO cells (DUKX-B11) (Chasin and Urlaub, 1980) were transfected with the ADA−DHFR expression plasmid (p9ADA5-29) and selected initially for ADA expression and were then amplified in increasing concentrations of 2′-deoxycoformycin as described (Kaufman *et al.*, 1986a). Two clones 1−0.1 and 2−0.1 selected for resistance to 0.1 μM 2′-deoxycoformycin and the same clones selected for further resistance to 100 μM 2′-deoxycoformycin are designated 1−100 and 2−100, respectively, and have been described previously (Kaufman *et al.*, 1986a). Clone E6−0.1 is a DHFR-positive line selected to 0.1 μM MTX resistance after transfection of DHFR-deficient CHO cells with p91023(B).

pCSF-1 was introduced into DHFR-deficient CHO cells, DUKX-B11, by protoplast fusion as described by Sandri-Goldin *et al.* (1981). *E. coli* HB101 cells harboring pCSF-1 were grown in 50 ml of M9 salts containing 0.5% (w/v) casamino acids, 0.4% (w/v) glucose, 0.012% (w/v) $MgSO_4$, 5 μg/ml thiamine and 10 μg/ml tetracycline to an A_{600} of 0.6. Chloramphenicol was added to 250 μg/ml and the culture incubated at 37°C for an addition 16 h in order to amplify the plasmid copy number. A suspension of protoplasts was prepared as described by Sandri-Goldin *et al.* (1981), added to CHO DUKX-B11 cells at a ratio of $\sim 1-2 \times 10^4$ protoplasts/cell, and centrifuged onto the cells at 2000 r.p.m. for 8 min in an IEC Model K centrifuge. After centrifugation, the supernatant was removed by aspiration and 2 ml of polyethylene glycol solution [150 g of PEG 1450 (Baker Chem. Co.) in 50 ml of medium] was added to each plate. Cells were centrifuged again at 2000 r.p.m. for 90 s, the PEG solution removed, and the plates rinsed three times in alpha medium containing 10% (v/v) fetal calf serum. Cells were then trypsinized and plated into tissue culture dishes in medium containing 100 μg/ml of kanamycin, 10 μg/ml each of thymidine, adenosine, deoxyadenozine, penicillin and streptomycin. Two days later the cells were trypsinized and subcultured 1:15 into media with 10% dialyzed fetal calf serum, penicillin and streptomycin, but lacking the nucleosides. Seven independent colonies that appeared after 12 days were trypsinized and combined into a single pool and grown in alpha medium lacking nucleosides (Kaufman *et al.*, 1985). These cells were subsequently grown in stepwise increasing concentrations of MTX starting at 0.02 μM, with sequential increases to 0.1, 0.5 and 2.0 μM, in order to obtain cells which had amplified the copy number of the GM-CSF gene. The selected population in 0.5 μM MTX was cloned by dilution plating in microtitre plates in alpha medium containing 10 μg/ml of penicillin, streptomycin and 10% (v/v) dialyzed fetal calf serum with 0.5 μM MTX. Clones obtained in 0.5 μM MTX were expanded and subsequently selected for growth in 2.0 μM MTX. Cells propagated in 0.02, 0.1, 0.5 and 2.0 μM MTX produced 2000, 4000, 12 000 and 50 000 units/ml/10^6 cells/day of GM-CSF, respectively, as determined by the colony formation assay with the KG-1 human myeloid leukemia cell line as described (Wong *et al.*, 1985).

Protoplast fusion yielded greater transfection efficiencies than $CaPO_4$-mediated DNA transfection and were necessary in order to obtain the rare transformants that efficiently express the polycistronic transcript. The inability to select for DHFR expression from polycistronic vectors in previous experiments (Kaufman *et al.*, 1985) was due to inefficient methods of DNA transfer.

Analysis of GM-CSF, ADA and DHFR expression

GM-CSF, ADA and DHFR synthesis were monitored by labeling of either transfected COS cells or CHO cells for 1 h with 100 μCi of L[^{35}S]methionine (sp. act. >8000 Ci/mmol, New England Nuclear). Cell extracts were prepared and analysed by SDS−polyacrylamide gel electrophoresis, either before or after immunoprecipitation with a rabbit anti-human GM-CSF antibody (kindly provided by E.Wang, Genetics Institute), a sheep anti-mouse ADA antibody (Ingolia *et al.*, 1985), or a rabbit anti-mouse DHFR antibody. *Staphylococcus aureus* was used as the immunoadsorbant as described (Kaufman and Sharp, 1982a). The ADA and DHFR antisera were kindly provided by Rodney Kellems, Baylor University. Gels were fixed in 30% methanol, 10% acetic acid and 10% trichloroacetic acid,

and prepared for fluorography by treatment with EnHance (New England Nuclear Corp.), and dried. Dried gels were autogradiographed with Kodak XAR-5 film on a Du Pont Cronex Lightning-Plus screen. Protein levels were quantitated by visual comparison of band intensities from multiple autoradiograms of different exposure times.

Total RNA was prepared by guanidine thiocyanate extraction (Chirgwin *et al.*, 1979) and was examined by blot hybridization (Thomas, 1980) following electrophoresis on formaldehyde–formamide denaturing agarose gels and transfer to nitrocellulose as described by Derman *et al.* (1981). Hybridization was carried out using gel-isolated restriction fragments which had been labeled with [α-^{32}P]dATP by nick translation (Rigby *et al.*, 1977). The DHFR probe was derived from a *Pst*I digestion of pDHFR26 (Chang *et al.*, 1978) and the GM-CSF probe was derived from an *Eco*RI digestion of pCSF-1 (Wong *et al.*, 1985). Both these probes contain the intact coding region for the respective polypeptides.

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References

Bos,J.L., Polder,L.J., Bernards,R., Schrier,P.I., van den Elsen,P.J., van der Eb,A.J. and van Ormondt,H. (1981) *Cell*, **27**, 121–131.

Chang,A.C.Y., Nunberg,J.H., Kaufman,R.J., Erlich,H.A., Schimke,R.T. and Cohen,S.N. (1978) *Nature*, **275**, 617–624.

Chasin,L.A. and Urlaub,G. (1980) *Proc. Natl. Acad. Sci. USA*, **78**, 5334–5338.

Chirgwin,J.M., Przybyla,A.E., MacDonald,R.J. and Rutter,W.J. (1979) *Biochemistry*, **18**, 5294–5299.

Clerx-van Haaster,C., Akashi,H., Auperin,C. and Bishop,D. (1982) *J. Virol.*, **41**, 119–128.

Derman,E., Krauter,K., Walling,L., Weinbergern,C., Ray,M. and Darnell,J.E. Jr. (1981) *Cell*, **23**, 731–739.

Dixon,L. and Hohn,T. (1984) *EMBO J.*, **3**, 2731–2736.

Ghosh,P.K., Reddy,V.B., Swinscoe,J., Lebowitz,P. and Weissman,S.M. (1978) *J. Biol. Chem.*, **253**, 3643–3647.

Hagenbuchle,O., Tosi,M., Schibler,U., Bovey,R., Wellauer,P.K. and Young, R.A. (1981) *Nature*, **289**, 643–646.

Haynes,J. and Weissman,C. (1983) *Nucleic Acids Res.*, **11**, 687–706.

Hendy,G., Kronenberg,H., Potts,J., Jr. and Rich,A. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 7365–7369.

Herman,R.C. (1986) *J. Virol.*, **58**, 797–804.

Hughes,S., Mellstrom,K., Koslik,E., Tamanoi,R. and Brugge,J. (1984) *Mol. Cell. Biol.*, **4**, 1738–1746.

Ingolia,D.E., Yeung,C.-Y., Orengo,I.F., Harrison,M.L., Frayne,E.G., Rudolph, F.B. and Kellems,R.E. (1985) *J. Biol. Chem.*, **260**, 13261–13267.

Kaufman,R.J. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 689–693.

Kaufman,R.J. and Sharp,P.A. (1982a) *J. Mol. Biol.*, **159**, 601–621.

Kaufman,R.J. and Sharp,P.A. (1982b) *Mol. Cell. Biol.*, **2**, 1304–1319.

Kaufman,R.J., Sharp,P.A. and Latt,S.A. (1983) *Mol. Cell. Biol.*, **3**, 699–711.

Kaufman,R.J., Wasley,L.C., Spiliotes,A.J., Gossels,S.D., Latt,S.A., Larsen,G.R. and Kay,R.M. (1985) *Mol. Cell. Biol.*, **5**, 1750–1759.

Kaufman,R.J., Murtha,P., Ingolia,D.E., Yeung,C.-Y. and Kellems,R.E. (1986a) *Proc. Natl. Acad. Sci. USA*, **83**, 3136–3140.

Kaufman,R.J., Wasley,L.C., Furie,B.C., Furie,B. and Shoemaker,C.B. (1986b) *J. Biol. Chem.*, **261**, 9622–9268.

Kitajewshi,J.K., Schneider,R.J., Safer,B., Munemitsu,S.M., Samuel,C.E., Thimmappaya,B. and Shenk,T. (1986) *Cell*, **45**, 195–200.

Kitamura,N., Semler,B.L., Rothberg,P.G., Larsen,G.R., Adler,C.J., Dorner, A.J., Emini,E.A., Hanacak,R., Lee,J.J., van der Werf,S., Anderson,C.W. and Wimmer,E. (1981) *Nature*, **291**, 547–553.

Konarska,M., Filipowicz,W., Domday,H. and Gross,H.J. (1981) *Eur. J. Biochem.*, **114**, 221–227.

Kozak,M. (1979a) *Nature*, **280**, 82–85.

Kozak,M. (1979b) *J. Biol. Chem.*, **254**, 4731–4738.

Kozak,M. (1980a) *Cell*, **22**, 7–8.

Kozak,M. (1980b) *Cell*, **22**, 459–468.

Kozak,M. (1984) *Nucleic Acids Res.*, **12**, 3873–3893.

Kozak,M. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2850–2854.

Kozak,M. and Shatkin,A.J. (1978) *J. Biol. Chem.*, **253**, 6568–6577.

Lau,Y.-F., Lin,C.C. and Kan,Y.W. (1984) *Mol. Cell. Biol.*, **4**, 1469–1475.

Liu,C.-C., Simonsen,C.C. and Levinson,A.D. (1984) *Nature*, **309**, 82–85.

Luthman,H. and Magnusson,G. (1983) *Nucleic Acids Res.*, **11**, 1295–1308.

Marsden,H.S., Haarr,L. and Preston,C.M. (1983) *J. Virol.*, **46**, 434–445.

McCormick,F., Trahey,M., Innis,M., Dieckmann,B. and Ringold,G. (1984) *Mol. Cell. Biol.*, **4**, 166–172.

Mellon,P., Parker,V., Gluzman,Y. and Maniatis,T. (1981) *Cell*, **27**, 279–288.

Mertz,J.E., Murphy,A. and Barkan,A. (1983) *J. Virol.*, **45**, 36–46.

O'Malley,R.P., Mariano,T.M., Siekierka,J. and Mathews,M.B. (1986) *Cell*, **44**, 391–400.

Peabody,D.S. and Berg,P. (1986) *Mol. Cell. Biol.*, **6**, 2695–2703.

Peabody,D.S., Subramani,S. and Berg,P. (1986) *Mol. Cell. Biol.*, **6**, 2704–2711,

Pelletier,J. and Sonenberg,N. (1985) *Cell*, **40**, 515–526.

Perler,H., Efstratiadis,A., Lomedico,P., Gilbert,W., Kolodner,R. and Dogson,J. (1980) *Cell*, **20**, 555–566.

Reichel,P.A., Merrick,W.C., Siekierka,J. and Mathews,M.B. (1985) *Nature*, **313**, 196–200.

Rigby,P.W., Dieckmann,M., Rhodes,C. and Berg,P. (1977) *J. Mol. Biol.*, **113**, 237–251.

Sandri-Goldin,R.M., Goldin,A.L., Levine,M. and Glorioso,J.C. (1981) *Mol. Cell. Biol.*, **1**, 743–752.

Scahill,S.J., Devos,R., Heyden,J.V. and Fiers,W. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 4654–4658.

Shatkin,A.J. (1976) *Cell*, **9**, 645–653.

Sompayrac,L.M. and Dana,K.J. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 7575–7578.

Svensson,C. and Akusjarvi,G. (1984) *Mol. Cell. Biol.*, **4**, 736–742.

Svensson,C. and Akusjarvi,G. (1985) *EMBO J.*, **4**, 957–964.

Swanstrom,R., Varmus,H.E. and Bishop,J.M. (1982) *J. Virol.*, **41**, 535–541.

Thomas,P.S. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 5201–5205.

Vogeli,G., Ohkubo,H., Sobel,M.E., Yamada,Y., Pastan,I. and de Crombrugghe, B. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 5334–5338.

Wengler,G., Wengler,G. and Gross,H.J. (1979) *Nature*, **282**, 754–756.

Yeung,C.-Y., Ignolia,D.E., Roth,D.B., Shoemaker,C., Al-Ubaidi,M.R., Yen, J.-Y., Ching,C., Bobonis,C., Kaufman,R.J. and Kellems,R.E. (1985) *J. Biol. Chem.*, **260**, 10299–10307.

Yoo,O.J., Powell,C.T. and Agarwal,K.L. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 1049–1053.

Wong,G.G., Witek,J.S., Temple,P.A., Wilkens,K.M., Leary,A.C., Luxenberg, D.P., Jones,S.S., Brown,E.L., Kay,R.M., Orr,E.C., Shoemaker,C.S., Golde, D.W., Kaufman,R.J., Hewick,R.M., Wang,E.A. and Clark,S.C. (1985) *Science*, **228**, 810–815.

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