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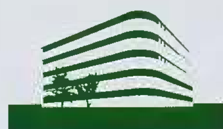

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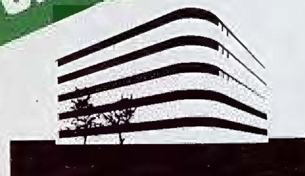

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The Regulation of Translation in Animal Virus-Infected Cells

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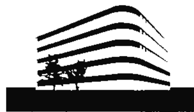

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J.W.B. Hershey - Post-translational modifications of initiation factors control translation rates.

W.C. Merrick - Translation of bicistronic mRNA constructs.

V. Agol - Towards understanding the mechanism of the internal binding of ribosomes to picornavirus RNAs.

N. Sonenberg - Structural and functional analysis of the ribosome landing pad of poliovirus: in vivo and in vitro translation studies

E. Wimmer - Function of the IRES element of picornaviruses.

R.J. Jackson - The novel mechanism of initiation of translation of picornavirus RNAs.

D.J. Rowlands - Translation of foot and mouth disease virus RNA.

P. Sarnow - Translation initiation by internal ribosome binding of viral and cellular mRNA molecules in eukariotic cells.

2. Tuesday 25:

H.O. Voorma - eIF-4F dependence of translation of mono and bicistronic messengers.

E. Ehrenfeld - Translation of cellular and viral mRNAs in poliovirus-infected cells.

D. Etchison - Alterations in proteins involved in translation in poliovirus-infected cells

L. Carrasco - The inhibition of host protein synthesis by picornaviruses.

A.J. Shatkin - Translational controls in cells expressing reovirus genes.

R.F. Garry - HIV-induced alterations of plasma membrane function: selective uptake of "impermeant" translational inhibitors.

M.J. Clemens - Control of translation by the Epstein-Barr virus small RNAs EBER-1 and EBER-2.

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R. Bablanian - Mechanism of selective translation of vaccinia virus MRNAS: Differential role of poly(A) and initiation factors in the translation of viral and cellular mRNAS.

3. Wednesday 26:

M.B. Mathews - Control of initiation in adenovirus infected cells.

R.J. Schneider - Suppression of cellular protein synthesis by adenovirus involves inhibition of cap-binding protein complex activity.

A.G. Hovanessian - Interferon-induced and double-stranded RNA activated proteins as enzymes in regulating cellular protein synthesis.

M.G. Katze - Regulation of the interferon-induced dsRNA activated protein kinase in animal virus-infected cells.

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FIRST SESSION

J. W. B. HERSHEY
W. C. MERRICK
V. AGOL
N. SONENBERG
E. WIMMER
R. J. JACKSON
D. J. ROWLANDS
P. SARNOW

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POST-TRANSLATIONAL MODIFICATIONS OF INITIATION FACTORS CONTROL TRANSLATION RATES

Hershey, J.W.B., Choi, S-Y., Scherer, B.J., Fabbri, B.J., *Davies, M.V., *Kaufman, R.J., Schwelberger, H.G. and Kang, H-A.

Department of Biological Chemistry, School of Medicine, University of California, Davis, Ca 95616, USA; *Genetics Institute, Inc. Cambridge, MA 02140, USA.

The phosphorylation of translational components leads to either the activation or inhibition of protein synthesis rates. Phosphorylation of eIF-2 leads to inhibition of global protein synthesis by preventing an efficient exchange of GDP for GTP on eIF-2 and thus inhibiting the binding of Met-tRNA to 40S ribosomal subunits. We have devised experiments to demonstrate that eIF-2 phosphorylation is the primary cause of translation inhibition in intact cells, by overexpressing mutant forms of eIF-2α cDNA in transfected cells. Inhibition of translation of either Adenovirus- or plasmid- derived mRNAs by the eIF-2α kinase, DAI, is mitigated by overexpression of mutant eIF-2α subunits where the putative targets of phosphorylation, Ser^{48} or Ser^{51}, are altered to Ala. Substitution of Ala for Ser^{51} prevents eIF-2α phosphorylation in the endogenous eIF-2 complex formed by an efficient exhange with the free, overproduced mutant eIF-2α on Ser^{51}, but reduces the negative effect of phospho Ser^{51} by an unknown mechanism. Using these methods, we show that eIF-2α phosphorylation contributes to the inhibition of protein synthesis seen in mammalian cells stressed by heat. The phosphorylation of eIF-4F, eIF-4B and eIF-3 result in activation of translation, possibly by altering their interactions with mRNA and with one another.

eIF-5A (Formerly eIF-4D) is uniquely modified by spermidine to form a lysine derivative, hypusine. We have cloned two yeast genes that encode eIF-5A and have shown that at least one of these must be active to support cell growth. Purified yeast eIF-5A functions in the mammalian assay for methionyl-puromycin formation, and the human cDNA complements the double null mutation in yeast. A mutant form where Arg replaces Lys^{51}, the site of hypusination, accummulates in yeast cells and is not modified, as expected. This Lys^{51} Arg mutant fails to function in yeast, suggesting that the hypusine modification is essential for the function of eIF-5A *in vivo*.
(Supported in part by NIH grant GM22135).

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TRANSLATION OF BICISTRONIC mRNA CONSTRUCTS

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Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, Ohio, U.S.A. 44106-4935

In the past few years, evidence has accumulated that indicates that there are alternate schemes to the standard Kozak scanning hypothesis for the initiation of translation. One such scheme is referred to as internal initiation and suggests that binding of the 40S subunit is to an internal portion of the mRNA, not at it's 5' end. The best studied examples are polio virus and EMC virus. The second scheme is termed re-initiation and proposes that the 40S subunit remains attached to the mRNA after termination of an upstream open reading frame (ORF) and scans down the mRNA to initiate a second reading frame. The system most studied to date and consistent with this mechanism has been the translationally controlled expression of GCN4 mRNA in yeast. In order to test these non-traditional initiation schemes for initiation factor requirements, we have used several different bicistronic mRNAs which are illustrated in the figure on the next page.

These constructs were translated *in vitro* using a nuclease treated reticulocyte lysate supplemented with initiation factors and/or m^7GTP. Several simple observations were made. First, the effect of added factors on internal initiation (translation of ORF-2) was qualitatively similar to the translation of ORF-1 in an uncapped mRNA. Second, added eIF-4F and/or eIF-4B preferentially enhanced the translation of ORF-2 in capped

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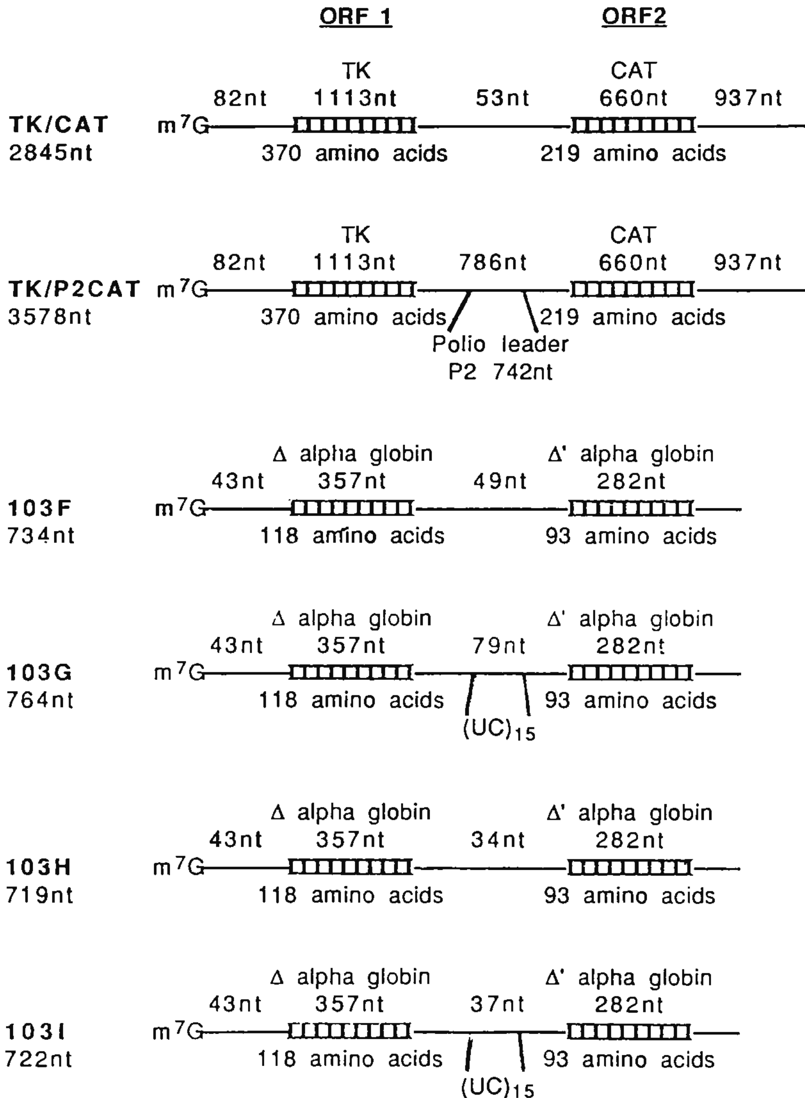
mRNA Constructs



Full length alpha globin = 142 amino acids with N-terminal methionine

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mRNAs and both ORF-1 and ORF-2 in uncapped mRNAs. These general observations were interpreted to indicate that both eIF-4F and eIF-4B participate in internal initiation, but that usually m^7G cap-dependent initiation is more efficient and consequently the mRNA specific factors tend to be sequestered at the 5' end of capped mRNAs.

A second series of experiments examined the interaction of translation factors with ribosomes as monitored by gel filtration. In contrast to previous studies which used sucrose gradients for analysis and found either eIF-2 (as the ternary complex) or eIF-3 associated with 40S subunits, most of the individual factors were found associated to some degree with the salt-washed ribosomes (40S, 60S or 80S ribosomes were not resolved). While these represent highly artificial studies (i.e. possible competing proteins aren't present), several possible new interpretations are:

1. The mRNA specific factors (especially eIF-4F) could bind to the 40S subunit before binding the mRNA.
2. rRNA is likely to be part of the site on the ribosome recognized by the translation factors (not just protein-protein interactions).
3. eIF-4B appears to contain a ribosome-dependent ATPase activity and is therefore a likely candidate to facilitate mRNA scanning.

This work was supported in part by a medical scientist training grant (NIH T32-GM-07520 to D.D.A.) and an NIH research grant (NIH GM-26796 to W.C.M.).

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TOWARDS UNDERSTANDING THE MECHANISM OF THE INTERNAL BINDING OF RIBOSOMES TO PICORNAVIRUS RNAs

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Institute of Poliomyelitis and Viral Encephalitides, the USSR Academy of Medical Sciences, Moscow Region 142782; and Moscow State University, Moscow 119899, USSR

The initiation of translation on picornavirus RNA templates is accomplished through cap-independent internal binding of ribosomes. Data will be reported strongly suggesting that two appropriately spaced *cis*-acting elements in the 5'-untranslated region of the viral RNA, an oligopyrimidine block (UUUCC) and a cryptic AUG, are involved in the template/ribosome recognition. Extended oligonucleotide linkers (23-46 bases long) were inserted into the full-length viral RNA just downstream of the UUUCC element. This resulted in a considerable decrease in the RNA *in vitro* protein-synthesizing activity. In several cases, viable insertion mutants have been obtained, but the viruses exhibited a decreased growth potential. Upon their reproduction in the cultured cells, the original mutants were very soon displaced by pseudorevertants, demonstrating a (partial) restoration of the both reproductive capacity of the virus and template activity of its RNA. Strikingly diverse genetic alterations were found within, or around, the inserts. With no exceptions, however, the revertant genomes had either (i) deletions (with diverse boundaries) inside the inserts (sometimes also extending into nearby viral nucleotides), or (ii) AUG-generating point mutations (again at different positions inside, or around, the inserts). In both cases, the proper arrangement of the UUUCC/AUG tandem was restored. Sometimes, the revertants also acquired additional point mutations resulting in the destabilization of the secondary

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structure around the relevant AUG. Taking into account that, in the natural entero- and rhinoviruses, both the UUUCC and the cryptic AUG at position 586 with the surrounding bases, exhibit complementarity to the 18S ribosomal RNA, it is proposed that the internal binding of ribosomes involves direct interaction of their RNA with the viral template; in other words, we are considering the oligopyrimidine tract as an analog of the prokaryotic oligopurine Shine-Dalgarno sequence. However, our results demonstrated that, in the distinction with the prokaryotic systems, the position of the UUUCC sequence relative to the preceding *cis*-element should be strongly fixed and the AUG triplet involved should not necessarily serve as the initiation codon.

To verify the significance of the combination of two appropriately spaced elements, UUUCC and AUG (placed within the proper context), a specially designed, cryptic AUG-containing linker was inserted downstream of the natural UUUCC; as a result, large-plaque-forming progeny was obtained just upon the primary transfection. Moreover, a dead construct lacking the entire conserved secondary structure domain encompassing the natural cryptic AUG, could be revived by inserting, in place of this domain, the above AUG-containing non-viral linker. Implications of the observed phenomena for the mechanism of the internal initiation on the picornaviral templates will be discussed.

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STRUCTURAL AND FUNCTIONAL ANALYSIS OF THE RIBOSOME LANDING PAD OF POLIOVIRUS: *IN VIVO* AND *IN VITRO* TRANSLATION STUDIES

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The naturally uncapped genomic and messenger RNAs of poliovirus initiate translation by an internal ribosome binding mechanism. The mRNA 5' untranslated region (UTR) of poliovirus is approximately 750 nucleotides in length and has six to eight (depending on the serotype) AUG codons upstream of the initiator AUG. The sequence required for internal ribosome binding has been termed "Ribosome Landing Pad" (RLP) or "Internal Ribosome Entry Site" (IRES). To better understand the mechanisms of internal initiation we have determined the boundaries and critical elements of the RLP of poliovirus type 2 (Lansing strain) in vivo. By using deletion analysis we demonstrate the existence of a core RLP in the poliovirus mRNA 5' UTR whose boundaries are between nucleotides 134 to 155 at the 5' end and nucleotides 556 to 585 at the 3' end. Sequences flanking the core RLP effect translational activity. The importance of several stem-loop structures in the RLP for internal initiation has been determined. Mutation of the phylogenetically conserved loop sequences in the proximal stem-loop structure of the RLP (nucleotides 127-165) abolished internal translation. However, deletion of the second stem-loop in the RLP (nucleotides 189-223) reduced internal translation by only 50%. Internal deletions encompassing nucleotides 240 to 300, 350 to 380, or 450 to 480, predicted to disrupt the third and fourth stem-loop structures in the RLP, also abrogated internal initiation. Small point mutations within a short polypyrimidine sequence, highly conserved among all picornavirus abolished translation. A conservation of distance between the conserved polypyrimidine tract and a downstream AUG could play an important role in the mechanism of internal initiation.

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FUNCTION OF THE IRES ELEMENT OF PICORNAVIRUSES

Eckard Wimmer, Christopher Hellen, Sung Key Jang, Mark Litterst, Aktheruzzamman Molla, Aniko Paul, Tatjana Pestova, and Gary Witherell; Department of Microbiology, School of Medicine, SUNY at Stony Brook, Stony Brook NY 11794, USA

The internal ribosomal entry site (IRES) of the picornavirus genome is an extended sequence up to 420 nucleotides long, that is located in the 5' non-translated region (5'NTR) and confers to picornavirus mRNA the property of cap-independent translation and internal ribosomal entry. We are focusing our studies on IRES elements of encephalomyocarditis virus (EMCV) and poliovirus, type 1 (PV1). We have defined the borders of the IRES element in EMCV, and we are studying the contribution of structural elements of two IRES elements to their function in vivo and in vitro. Two subjects are of particular interest to us:

(i) The binding to the IRES, and the function, of the novel cellular RNA binding protein protein, p57. This protein is essential for translation in vitro of both EMCV and PV1 mRNAs, but it is non-essential for translation of globin mRNA. Accordingly, p57 does not bind to globin mRNA. On the other hand, we have observed that p57 can bind to more than one structural element of the EMCV IRES. In PV1 5'NTR we have so far determined a single binding site for p57.

(ii) The selection of the initiating AUG codon within the poliovirus and EMCV 5'NTRs. Through the construction of point mutations and deletions we have come to the conclusion that an AUG triplet preceded by an oligopyrimidine (Yn) tract is essential for IRES function in EMCV and PV1 RNA, regardless of whether the AUG triplet serves as initiating codon for polyprotein synthesis. Whereas in EMCV the Yn-Xm-AUG unit (where Xm generally in picornavirus RNA is a segment of 15 to 25 nucleotides separating Yn from the AUG) contains the initiating AUG, in PV1 5'NTR this unit is located 154 nt upstream from the initiating AUG. The reason that the Yn-Xm-AUG unit of PV1 is not used in translation is the poor context of its AUG (CUU AUG G). Conversion of this context to ACC AUG G "activates" this AUG which leads to the synthesis of a 6kD protein, as expected from the predicted reading frame in this region. Introduction of this mutation into PV1 cDNA leads to a mutant virus with a small plaque phenotype. Based on these and other data we will present

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a unifying hypothesis as to the selection of the site in picornavirus 5'NTR where polyprotein synthesis commences.

Mutations (deletions, insertions, point mutations) in Yn-Xm-AUG or in sequences preceding this unit produced an unexpected complexity of in vitro phenotypes. It is clear that the sequences immediately upstream from the Yn-Xm-AUG unit of EMCV are also crucial for IRES function. Whereas most structural elements of the EMCV IRES seem important for function in dicistronic constructs in vitro, we have found one site that tolerates insertions as long as 120 nt.

Using the propenstity of IRES elements to promote internal ribosomal entry we have constructed a dicistronic poliovirus genome with the novel property of two independently regulated reading frames. A viable poliovirus was generated with the genotype: [PV1]5'NTR-P1-[EMCV]5'NTR-[PV1]P2-P3-3'NTR. The properties of this mutant, which has a small plaque phenotype, and the properties of derivatives thereof, will be discussed.

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THE NOVEL MECHANISM OF INITIATION OF TRANSLATION OF PICORNAVIRUS RNAs

Richard, J. Jackson, Andrew Borman, Michael T. Howell, and Ann Kaminski.

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CD2 1QW, U.K.

The 5'-untranslated region (5'-UTR) of animal picornaviruses contain a segment of some 450-500 nt. that acts as an internal ribosome entry site (IRES). The initiation of translation of these RNAs takes place by a mechanism in which ribosomes bind directly to this IRES rather than scan the RNA from the physical 5'-end, as is thought to occur with all capped mRNAs. In respect of sequence homology in the IRES elements, the main picornavirus genera fall into two groups: (i) entero- and rhinoviruses; (ii) foot and mouth disease virus and the cardioviruses. In the second group, the IRES is the ~450 nt. immediately upstream of the authentic initiation codon; whereas the IRES of the entero-/rhinoviruses extends from about nt. 100 to nt. 560, and does not abut the authentic initiation site. A further difference between the two groups is that translation of cardiovirus RNAs in the rabbit reticulocyte lysate system is highly efficient, whereas the entero-/rhinovirus RNAs are very inefficient. However, the entero-/rhinovirus RNAs are efficiently translated in extracts from HeLa cells, and factors can be isolated from HeLa cells which, when added to reticulocyte lysates, greatly stimulate the translation of these RNAs.

We have examined the local determinants of the ribosome entry site of cardiovirus RNAs, by using clones in which the 5'-UTR of encephalomyocarditis (EMCV) RNA are fused to an appropriate reporter gene in vectors carrying a bacteriophage T7 RNA polymerase promoter. *In vitro* transcripts of these clones were translated in the rabbit reticulocyte lysate. The results show that ribosomes initiate translation almost exclusively at the authentic initiation site (AUG-11), and ignore a nearby out-of-frame AUG codon (AUG-10) which is located 8 nt. upstream of AUG-11 (and is found only in a some, but not all, cardiovirus strains). This result argues strongly that ribosome 'enter' and bind directly at AUG-11, and do not select this AUG by scanning from an usptream entry site. An analysis of the translation of deletion and insertion mutants suggests that the internal entry mechanism selects AUG-11 rather than AUG-10, partly on the basis of hte distance of AUG-11 from upstream motifs, and in part because of local sequence features around AUG-11 that have not yet been fully identified.

The same general experimental approach has been used to map the limits of the IRES elements in both poliovirus (an enterovirus) and in human rhinovirus. In these experiments, dicistronic constructs were used

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in which the viral 5'-UTR (or deletion mutants of this UTR) was inserted between the two cistrons, and the influence of this insertion on the translation of the downstream cistron was examined. When the rhinovirus 5'-UTR was used, translation of the downstream cistron was almost completely dependent on supplementing the reticulocyte lysate with HeLa cell factors. The 3'-boundary of the rhinovirus IRES was mapped to a region between nt. 554 and 562; a deletion from the 3'-end of the UTR to nt. 562 allowed efficient translation of the downstream cistron, whereas a deletion to nt. 554 did not.

Similar results were obtained using the 5'-UTR of poliovirus type 1, but in this case there is the complication of a second, additional, IRES element extending from a 5'-boundary mapped to between nt. 579 and 595 and a 3'-boundary located upstream of nt. 670. This second IRES element can promote translation of the downstream cistron of dicistronic mRNAs in the reticulocyte lysate system, but as such translation is not responsive to HeLa cell factors, there is some question as to whether this second IRES has physiological significance. The physiologically relevant IRES in entero-/rhinovirus therefore seems to extend from about nt. 100 to about nt. 560. This IRES promotes ribosome entry at about nt. 570 - 580, and from this entry site the ribosomes appear to scan the RNA in the usual 5'-3' direction, following the normal 'rules' of the scanning ribosome model, until the authentic initiation site is reached, at nt. 611 in rhinovirus, or nt. 743 in poliovirus.

Thus the models for internal entry on RNAs of the cardiovirus group and those of the entero-/rhinovirus group are essentially similar except that internal ribosome entry is followed by scanning only in the case of the entero-/rhinoviruses. The essential features of the IRES in all picornavirus seem to be a complex secondary/tertiary structure comprising at least 400 nt., followed by a pyrimidine rich tract, which is itself located some 25 nt. upstream of the actual ribosome entry site. In addition, sequences at the ribosome entry site itself may have a quite strong influence on the efficiency and the precise site of internal ribosome entry.

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TRANSLATION OF FOOT AND MOUTH DISEASE VIRUS RNA

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Several features of the structure and organization of the genome of FMDV serve to distinguish it from those of other picornaviruses. Instead of the single species of VPg found in other picornavirus, the 5' terminal nucleotide of each FMDV RNA is linked to one of three independently coded, different VPg molecules, the sequences of which are highly conserved across different serotypes of the virus. The 5' UTR is c 1300 nucleotides in length and includes a poly C tract of up to 200 nucleotides preceded by a region of c 400 nucleotides which appears to possess a high degree of secondary structure. To the 3' side of the poly C tract there is a region which can be folded into a number of pseudoknot domains, the number of these domains varying between different viruses. The remainder of the 5' UTR, between the pseudoknots and the initiating AUG, can be folded into a structure similar to that of the internal ribosome entry site of cardiovirus UTRs and has been shown to funciton as such when inserted between open reading frames in a bicistronic message. All FMDVs have two inframe initiation codons, separated by 81 nucleotides, both of which are functional in vitro and in vivo. Both initiating AUGs are closely preceded upstream by polyprimidine tracts which have been shown to be necessary for efficient translation. The sequences flanking the initiating AUGs vary between viruses but the second is always in a favourable 'Kozak' context. The first product(s) of translation, the leader(s) (Lab & Lb), has been shown to have proteolytic activity and cleaves itself from P1, the precursor to the structural proteins. It can also lead to the cleavage of host p220, with consequent inhibition of cap dependent mRNA translation.

Thus the translation of FMDV RNA displays a combination of the features of the translation of the entero and rhiboviruses on one hand and the cardioviruses on the other. The structure of the FMDV RNA 5' UTR resembles that of the cardioviruses and, like them, it is an extremely efficient mRNA in vitro. Unlike the cardioviruses, FMDV expresses a proteolytic activity which can result in p220 cleavage, as seen in the entero and rhinoviruses, albeit that the location of the protease on the genome is unique to FMDV. However, the kinetics of protein translation in FMDV infected cells do not show the dramatic shutt off of host protein synthesis

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seen with, for example, poliovirus, but simply swops from host to virus protein synthesis during the course of virus replication. Among the unanswered questions relating to the translation of FMDV RNA are; the significance (if any) of the pseudoknot domains, the function of the poly C tract and the preceding heteropolymeric region; why are there always two initiation sites? and what is the significance of the induction of p220 cleavage?.

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TRANSLATION INITIATION BY INTERNAL RIBOSOME BINDING OF VIRAL AND CELLULAR mRNA MOLECULES IN EUKARIOTIC CELLS.

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A ribosome scanning model has been proposed to explain the initiation of eukaryotic mRNAs. In this model, binding of the 43S ternary ribosomal subunit near or at 5' end of the mRNA is facilitated by a concerted interaction between the m' GpppN cap-structure at the end of the mRNA and the cap binding protein complex eIF-4F. However, picornaviral mRNAs do not possess a 5'terminal cap-structure and have been shown to be translated by internal ribosome binding.

We have been pursuing two questions: First, is translation by internal ribosome binding regulated in eukaryotic cells? Secondly, are there cellular mRNAs that can also be translated by internal ribosome binding?

We have observed that polioviral translation initiation by internal ribosome binding is enhanced in infected cells. To identify the viral transactivator, we cotransfected a reporter plasmid, containing the promoter for T7 RNA polymerase upstream of the 5' noncoding region of poliovirus linked to a firefly luciferase gene, together with effector T7-plasmids containing various parts of the poliovirus cDNA, into a cell line, OST7 (Elroy-Stein and Moss, PNAS 87: 6743-6747), that contains a stably integrated gene for T7 RNA polymerase. We observed that translation products derived from the effector plasmids enhanced translation of the reported mRNA. Using this assay, we have found that the viral polypeptide 2A is responsible for transactivation of polioviral RNA translation in mammalian cells.

We have found that three cellular mRNAs, encoding the immunoglobulin heavychain binding protein (BIP) and, and more recently, the homeotic *Antennapedia* (Antp) and *Ultrabithorax* (Ubx) proteins of *Drosophila melanogaster*, can be translated by internal ribosome binding in cultured cells. Specifically, we have shown that *in vitro*-synthesized dicistronic mRNAs containing various parts of the *Antp* and *Ubx* 5'NCRs as intercistronic spacers (ICS) between the chloramphenicol acetyltransferase (CAT) and luciferase (LUC) coding regions mediated the translation of the second LUC cistron by internal ribosome binding in cultured cells.

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The finding that 5' NCRs of two homeotic genes of Drosophila melanogaster are capable of translational initiation by internal ribosome binding explains several observations about the structure of these genes. For example, the 5' NCR of antennapedia mRNA contains 1740 nucleotides spanning three exons and includes 15 upstream AUG codons that are not used for translation initiation. We have identified a single exon containing 250 nucleotides that mediates internal ribosome binding. This sequence is highly conserved at the nucleotide level between different Drosophila species. The gene product of this mRNA, the antennapedia protein, regulates segmental indentity in the thorax of the fruit fly. Loss of antennapedia function can result in altered development of the segments in the thorax or can cause legs to be transformed into antennae. Currently, we are determining at what stages during the development in the fruit fly this unusual translational regulation may occur.

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SECOND SESSION

H. O. VOORMA
E. EHRENFELD
D. ETCHISON
L. CARRASCO
A. J. SHATKIN
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M. J. CLEMENS
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eIF-4F DEPENDENCE OF TRANSLATION OF MONO- AND BICISTRONIC MESSENGERS

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The translation initiation on capped cellular mRNAs depends on a number of initiation factors of which eIF-4A, eIF-4B and eIF-4F play a crucial role in the binding of the messenger to the 40S ribosomal subunit. These factors are capable to unwind the secondary structure of the 5' untranslated region, 5' UTR, of the mRNA, which facilitates the subsequent binding of the 43S preinitiation complex. eIF-2 takes part in the selection of the initiation region surrounding the AUG codon. The eIF-4E subunit of eIF-4F binds to the cap of the mRNA allowing the other factors to bind as well.

As a logical consequence of this eIF-4F function one may postulate that eIF-4F does not play a role in cap-independent translation. This problem has been encountered by the construction of a bicistronic mRNA, consisting of the 5' capped coding region of eIF-4AI, followed by the 5' UTR of EMC and the reporter gene CAT.

In order to measure the eIF-4F dependence of translation of these two cistrons, we included in the cell free translation system the messenger comprising the 5' UTR of FMDV followed by the coding region of the L' protease, that cleaves the p220 of eIF-4F. Translation of this L'-mRNA prior to the translation of the bicistronic mRNA results in complete inhibition of eIF-4A synthesis, the upstream cistron, whereas CAT-synthesis on the downstream cistron is virtually unchanged.

Another construct, comprising the adenovirus TPT-leader followed by the CAT gene has been translated under similar conditions.

From literature data it is known that late adenovirus messengers are translated in poliovirus-infected cells, suggesting a cap-independent translation. To our surprise the co-translation assay as described, with L'-mRNA and TPT-CAT-mRNA resulted in a severe inhibition of CAT synthesis indicating a dependence on intact p220 in these assays. In vitro translation also revealed a complete dependence of capped TPT-CAT-mRNAs on eIF-4F and indicates that no cap independent translation initiation takes place on TPT-carrying mRNAs.

It may mean that eIF-4F independent translation in vivo, in polio- and adenovirus double-infected cells is brought about by adeno- or poliovirus-encoded proteins.

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TRANSLATION OF CELLULAR AND VIRAL mRNAs IN POLIOVIRUS-INFECTED CELLS. Elizabeth Wyckoff, John Gebhard and Ellie Ehrenfeld, Department of Cellular, Viral and Molecular Biology, University of Utah School of Medicine Salt Lake City, UT 84132 U.S.A.

Poliovirus infection of HeLa cells causes a marked and rapid inhibition of host cell protein synthesis, which correlates with the cleavage of the p220 subunit of the translational initiation factor, eIF-4F. eIF-4F is a multi-subunit protein which binds to the m^7G cap group on the 5'ends of capped mRNAs, and which is involved in RNA unwinding, presumably to facilitate ribosome binding to the mRNA and scanning to the initiating AUG codon. The poliovirus-induced cleavage of the p220 subunit is thought to inactivate eIF-4F so that cellular mRNAs cannot be translated, while the uncapped poliovirus RNA is translated by a cap-independent mechanism. The poliovirus-encoded 2A protease is required for cleavage of p220, but does not itself cleave p220. A current model is that 2A activates an unidentified, cellular protease which then cleaves p220.

An assay was developed to identify additional proteins required for the 2A-induced cleavage of p220. Using this assay, we showed that an activity present in highly purified preparations of another translational initiation factor, eIF-3, is required for 2A-dependent cleavage of a partially purified p220 substrate. eIF-3 shows no detectable structural or functional modifications in poliovirus-infected cells, or after incubation with bacterial extracts that express active 2A protease. In addition, eIF-3 is also required for p220 cleavage catalyzed by partially purified preparations of the activated p220 cleavage activity in poliovirus-infected cells. The exact role of eIF-3 in the cleavage reaction is under investigation.

Inactivation of the cap recognition system is an effective means of specifically inhibiting host cell protein synthesis since poliovirus RNA is not capped, and therefore must initiate translation by another mechanism. Others have demonstrated that ribosome binding to picornavirus RNAs occurs via an internal entry site. It is highly likely that cellular or viral proteins assist in this form of ribosome binding. We have used UV crosslinking to detect Hela cell proteins that interact stably with the 5'noncoding region of poliovirus RNA, and to localize their binding sites on the RNA. At least four major protein-RNA complexes were identified. One binds RNA sequences or structures located between nucleotides 457-626; two interact with nucleotides 286-456; one was not mapped. The effects of these protein-binding domains on translation were measured by RNA competition assays. The results indicate that nucleotides 286-456 bind proteins that are specific for poliovirus translation and which are present in Hela cells but not in significant amounts in rabbit reticulocyte lysates. The downstream domain (nts 457-626) binds general translation factors, required for translation of all mRNAs, present in both rabbit reticulocytes and Hela cells.

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ALTERATIONS IN PROTEINS INVOLVED IN TRANSLATION IN POLIOVIRUS-INFECTED CELLS

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Monoclonal antibodies have been used to identify alterations in cellular proteins following poliovirus infection. Two such alterations have now been identified; cleavage of the p220 subunit of eIF-4F, and an alteration in an apparent cytoskeletal protein which partially co-purifies with RNA-binding activity and eIF-4B. We are using antibody approaches to study interactions of p220 with other initiation factors in order to determine its function. We are just beginning to characterize the cytoskeletal antigen and its possible relation to changes in translation.

Poliovirus-induced cleavage of a 220,000 kDa polypeptide, later determined to be a subunit of the cap-binding complex eIF-4F, was originally discovered using an antiserum which had been raised against a purified eIF-3 preparation. Co-purification suggested a complex between the two factors, an idea supported by the more recent demonstration that a cap-binding complex can be purified from uninfected cells which consists of both eIF-3 and eIF-4F polypeptides. No similar complex can be purified from infected cells. These results suggest that an eIF-3/4F complex might be a functional intermediate during translational initiation which mediates mRNA binding to the 40S ribosomal subunit and that p220 cleavage might dissociate this complex. To determine whether p220 forms a complex with eIF-3 on the 40S ribosomal subunit, cell lysates harvested at different times after infection were analyzed on sucrose gradients for eIF-4F and eIF-3 using monoclonal antibodies. ELISA and immunoblot detection of p220 and the p170 subunit of eIF-3 have confirmed that a portion of the p220 in uninfected cells is associated with the 40S subunit and that by 2 hrs post-infection cleaved p220, but not p170, is completely dissociated. A natural affinity of the eIF-3/4F complex for purified 40S subunits in the absence of other factors was demonstrated by a gradient shift assay of p220 and p170 antigens. Thus eIF-3 and eIF-4F indeed form a complex which readily binds to 40S subunits. The complex is labile, however, and can be dissociated by DEAE chromatography or by sedimentation on sucrose gradients in 500 mM salt. Association with 40S subunits appears to require a "pre-assembled" form of the eIF-3/4F complex. After separation of eIF-3 and eIF-4F by DEAE chromatography, p220 no longer shifts to the 40S region of sucrose gradients with eIF-3. Thus the complex between eIF-3 and eIF-4F is not due to non-specific interactions. Its formation is likely to be an early event in formation of the initiation complex containing capped mRNA which is abolished by cleavage of p220.

The monoclonal antibody approach to determining differences in host cell proteins following poliovirus infection has revealed another alteration. An antibody raised against a partially-purified eIF-4F preparation recognizes a heterogeneous antigen which becomes reduced in size in poliovirus-infected cells. Unlike p220, this antigen (referred to as 25 C C1) is not altered

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THE INHIBITION OF HOST PROTEIN SYNTHESIS BY PICORNAVIRUSES.

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Picornaviruses are good models to analyze strategies used by animal viruses to interphere with host protein synthesis. In this regard both poliovirus and EMC virus have been intensively studied. Different mechanisms may operate in the inhibition of host translation by these two viruses. Thus, EMC virus can inhibit host translation by altering membrane permeability to cations. In favor of this suggestion evidence was found in cell-free systems that ions are the only effectors able to increase translation of picornavirus mRNAs at the same time that block translation of most cellular mRNAs. In addition, a good correlation exists between the shut-off of host translation by EMC virus and the ionic modifications found in the cytoplasm of EMCV-infected cells.Moreover, in this system shut-off can be reversed by modifying the ionic concentration. Finally, agents that modify the cell membrane, like ionophores, inhibit cellular protein synthesis in intact cells, whereas viral translation is more resistant. Therefore, picornavirus mRNAs have evolved special structures in the non-coding sequence of their messenger in order to initiate translation by a mechanism which is more efficient under ionic conditions that are harmful to cellular translation

A cellular protein that forms part of the cap binding complex , p220, is cleaved after poliovirus infection. It has been hypothesyzed that this event is responsible for the shut-off of translation inferred by poliovirus. However, increasing evidence indicates the lack of correlation between p220 cleavage and the drop in protein synthesis. Thus, detailed kinetic analyses show that intact p220 cannot be detected at times when host translation is not inhibited. The use of selective inhibitors of poliovirus RNA replication provides an additional means to dissociate p220 cleavage from shut-off. Finally, conditions were found that prevented the inhibition of host translation by poliovirus, but still p220 was cleaved by poliovirus under those conditions. The contrary situation i.e. inhibition of cellular translation with no p220 cleavage has been reported for a poliovirus mutant in protease 2A (Bernstein et al., J. Virol. 5, 2913 (1985)). Therefore,additional studies are needed to know in detail both the exact mechanism and the poliovirus protein responsible for shut-off.

To identify the poliovirus protein involved in membrane modification and also the viral protein responsible for shut-off, we have cloned in eukaryotic vectors each individual poliovirus non-estructural protein. These proteins have already been efficiently expressed in *E. coli* systems.These constructions will be transfered to eukaryotic inducible vectors in order to express them upon induction in selected mammalian cell clones.Progress of this work will be presented.

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TRANSLATIONAL CONTROLS IN CELLS EXPRESSING REOVIRUS GENES

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The S1 gene in all three reovirus serotypes codes for two distinct polypeptides by translation of a single mRNA containing a short open reading frame nested within a longer one. In reovirus-infected cells and in cell-free systems the serotype 3 S1 mRNA species directs synthesis of a 49 Kd hemagglutinin (α 1) from the cap-proximal AUG located 13 nucleotides from the 5' end. The next AUG, 58 nucleotides further downstream, corresponds to the initiator codon of a non-structural polypeptide, p14. Bicistronic mRNAs are also utilized by a variety of other animal viruses including adeno-, myxo- and paramyxoviruses. In addition, some cellular mRNAs code for more than one protein by initiation at different AUG's. To determine how S1 mRNA would function in the absence of virus infection, mouse C127 cells were stably transformed with a DNA copy of the S1 gene. Both α1 and p14 were produced by translation of a full length transcript, demonstrating that uninfected animal cells have the capacity to express overlapping reading frames in a bicistronic mRNA.

Transient expression of the S1 gene in COS cells was used to assess the effects of initiation at the α1 AUG on the expression of the downstream, p14 reading frame. As predicted from the modified scanning model, minor sequence changes that diminished the consensus around the α1 initiator site enhanced the synthesis of p14. For example, DNA containing the α1 initiator in an optimal context, GCC GCC ACC ATGG, yielded barely detectable amounts of p14, and conversion of the sequence to GAA AAA GTT ATGG markedly increased p14 production. Maximal p14 levels were obtained in the absence of α1 synthesis, i.e. when the α1 AUG was mutated to UUG or a termination codon was inserted in the α1 reading frame before the p14 initiator. In contrast to p14 synthesis, the different initiation efficiencies at the α1 AUG were not reflected in the α1 polypeptide yields which were similar with either a weak or strong consensus. These results indicate that α1 synthesis is rate-limited at a level after initiation, e.g. during elongation due to limited availability of certain isoacceptor tRNAs. A regulatory effect of codon usage on bicistronic S1 mRNA translation is also suggested by the

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codon usage on bicistronic S1 mRNA translation is also suggested by the finding that the amounts of p14 relative to α1 were higher in reovirus-infected mouse L cells than in infected C127 or COS cells. The results suggest that during simultaneous translation of two reading frames in a single bicistronic mRNA, the ribosimes in one frame interfere with movement of ribosome in the other frame due to different rates of elongation. In S1 mRNA, the basis for *cis* regulation of p14 production by α1 synthesis may result from pausing of ribosomes in the less efficiently translated, longer reading frame.

The reovirus S4 gene has been implicated in a serotype-dependent inhibition of host translation in virus-infected L cells. S4 codes for polypeptide α3 which binds double-stranded (ds) RNA. As a consequence, α3 may regulate translation by blocking activation of DAI, the dsRNA-activated eIF-2α kinase. Consistent with this idea, the S4 gene effectively substituted for adenovirus VA1 in COS cells expressing dihydrofolate reductase under conditions that require inhibition of DAI activation. In addition, CAT mRNA translation was increased several-fold when COS cells were co-transfected with a DNA copy of the S4 gene derived from any of the three reovirus serotypes, presumably also due to diminished activation of DAI. Lysates of type 3 infected mouse L cells contained more α3 than type 2 extracts and required addition of 5-10 times more dsRNA to obtain maximal DAI phosphorylation. These results are consistent with the presence of higher levels of activated DAI and greater host translational shut off in cells infected at high multiplicity with type 2 as compared to type 3 (or 1) reovirus.

As an alternative to binding dsRNA, α3 can form 1:1 complexes with the reovirus M2-encoded polypeptide product, μ1C. Consequently, DAI-mediated translational regulation in reovirus-infected cells probably reflects the ratio of these two polypeptides, i.e. DAI activation can be prevented where S4 exceeds M2 expression and α3 is available to bind dsRNA. In agreement with this hypothesis, CAT stimulation by S4 in COS cells was decreased by M2 co-expression. In DNA transfected COS cells, as in reovirus-infected L cells, the M2 primary translation product, μ1, was N-terminally myristoylated. However, in COS cells μ1 was not cleaved to μ1C unless the S4 gene was co-expressed- -conditions which yielded α3-μ1C complexes. Analyses of site-specific mutants indicate that myristoylation and proteolytic processing are related. Like adeno, polio, influenza and other animal viruses, reoviruses apparently can regulate translation in infected cells by modulating the activation of DAI and the phosphorylation state of eIF-2α.

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HIV-INDUCED ALTERATIONS OF PLASMA MEMBRANE FUNCTION: SELECTIVE UPTAKE OF "IMPERMEANT" TRANSLATIONAL INHIBITORS

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Human immunodeficiency virus (HIV), the etiologic agent of the AIDS, is capable of inducing two types of cytopathic effects in cultured $CD4^+$ cells. One type of HIV-induced cytopathology involves fusion of the plasma membranes of infected cells with other $CD4^+$ cells. The multinucleated cells formed as a result of this process are incapable of maintaining viability due to an irreversible expansion in volume. A second type of HIV-induced cytopathology culminates in death of single cells. The envelope glycoproteins of HIV have been implicated in both cytopathic processes. The HIV transmembrane glycoprotein can be fitted to the scaffold of the 3Å structure of the influenza virus fusion protein HA2 while accounting for all known functional and immunological features (Figure 1).

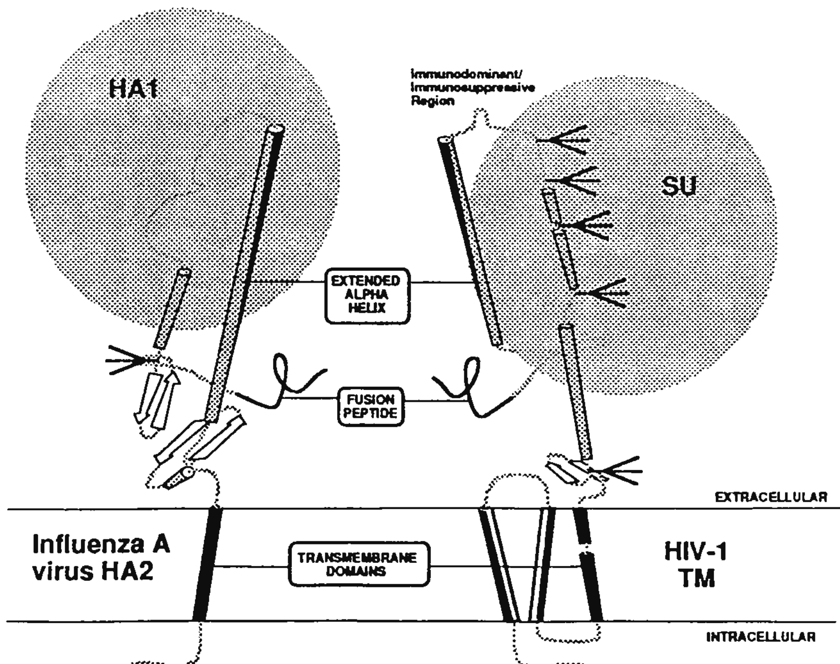

Figure 1. *Structural similarities between the transmembrane proteins of HIV and influenza virus.*

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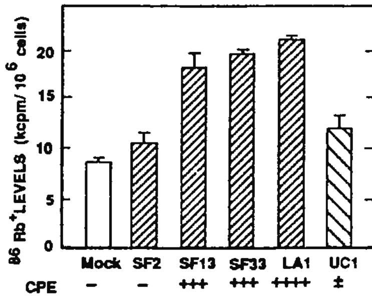

Figure 2. *Intracellular $^{86}Rb^{+}$ levels during infection with HIV variants which differ in cytopathic potential.* HuT 78 cells (RH9 subclone) were infected with HIV variants (HIV-1: SF2, SF13, SF33, LA1; HIV-2: UC1) previously demonstrated to differ in cytopathic potential or mock-infected. After 48 hrs the cells were labeled to equilibrium with $^{86}Rb^{+}$, and intracellular levels of the potassium tracer were quantitated. Standard error of the mean of triplicate samples is represented by the error bars. The results indicate that HIV variants with a high cytopathic potential (SF13, SF33, LA1) increase intracellular Rb^{+} relative to mock-infected cells to a greater extent than variants with lower cytopathic potential (SF2, UC1).

As with myxoviruses, insertion of HIV envelope proteins (either during direct virus-cell fusion or following *de novo* synthesis) may disrupt cell volume homeostasis and cell physiology by modifying plasma membrane "permeability" and altering intracellular ion concentrations. Ultrastructural and biophysical methods were used to investigate cell surface modifications that may result in cytopathology during infection of CD4+ T-lymphoblastoid cells. Cytoplasmic vacuolization occurred in some cells within the first hour after HIV infection, and this correlated with the numbers of virions bound at the cell surface. Penetration was exclusively by virion:plasma membrane fusion, and resulted in formation of distinct membrane discontinuities, pores, and "ballooning".

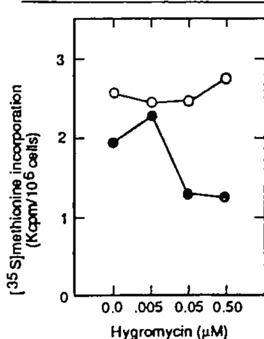

Figure 3. *Inhibition of protein synthesis in HIV-infected cells and mock-infected cells by Hygromycin, an "impermeant" inhibitor of protein synthesis.* HuT 78 cells (RH9 clone) were exposed to HIV or mock-infected. At 24 hours pi the cells were treated with various concentrations of hygromycin as show then labeled with [^{35}S]methionine. Incorporation of the labelled methionine into TCA precipitable material was then quantitated by scintillation counting. Hygromycin B, an aminoglycoside antibiotic, is relatively impermeant and therefore does not inhibit protein synthesis in mock-infected cells (open symbols). However this compound does inhibit protein synthesis in cells rendered more "permeable" by HIV (closed symbols).

Alterations of intracellular concentrations of sodium and potassium were also observed as an early consequence of cytopathic HIV infection (Figure 2). These results suggest that HIV may initiate processes that damage the cell membrane and culminate in death of a subset of infected cells. Furthermore, as a result of HIV-induced plasma membrane modifications certain translational inhibitors, such as the aminoglycoside hygromycin B, are selectively taken up into infected CD4^{+} lymphoblastoid cells (Figure 3). These compounds are relatively impermeant to uninfected cells, and therefore non-toxic. Compounds that are selectively transported into HIV-infected cells may be developed as AIDS drugs.

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CONTROL OF TRANSLATION BY THE EPSTEIN-BARR VIRUS SMALL RNAs EBER-1 AND EBER-2

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Epstein-Barr virus is a ubiquitous herpesvirus that is associated with a number of human malignancies; it can also efficiently immortalise human B lymphocytes in vitro. The majority of cells become latently infected and express only a very limited number of viral genes. Prominent among these are two small RNAs, EBER-1 and EBER-2, that are transcribed at equal rates but accumulate to different steady state levels due to differential rates of turnover. We have transcribed these RNAs in vitro using T7 bacteriophage RNA polymerase and have demonstrated that both species can play a role in the regulation of translational initiation. In the reticulocyte lysate system the inhibition of protein synthesis by double-stranded RNA is prevented by high concentrations of either EBER-1 or EBER-2. This effect is believed to be due to the ability of these RNAs to bind to the 62 kDa dsRNA-activated protein kinase DAI and to block the ability of this enzyme to phosphorylate initiation factor eIF-2. Although EBER-1 is 10-fold more abundant than EBER-2 in EBV-infected cells the two RNAs appear to have approximately equal affinities for DAI and to act over similar concentration ranges in vitro. Another small viral RNA, adenovirus VA_I RNA, behaves very similarly in these assays. In contrast, two small human cellular cytoplasmic RNAs, Y_1 and Y_5, do not rescue the inhibition of protein synthesis by DAI and do not compete with EBER-1 for binding to the kinase.

In addition to DAI, at least two other proteins bind EBER-1 - the 46.7 kDa La antigen and the recently described 14.8 kDa EAP protein. These proteins may be involved in the movement of EBER-1 between nucleus and cytoplasm, suggesting that the small viral RNAs may have an additional nuclear role.

It has been demonstrated that neither EBER-1 nor EBER-2 is required for productive EBV replication or for immortalisation of B lymphocytes by the virus in vitro. However, since DAI is induced by interferons in mammalian cells and acts as one of the enzymes involved in the cellular antiviral response, the ability of the EBERs to

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block its activation may be necessary for the virus to evade the host interferon system during the establishment of latent infections in vivo. It is therefore of interest that interferon treatment can itself affect the synthesis and stability of the EBERs in the EBV-positive Daudi cell line. The physiological significance of EBER-1 and EBER-2 expression and the structure-function relationships of these small RNAs will be discussed.

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MECHANISM OF SELECTIVE TRANSLATION OF VACCINIA VIRUS MRNAS: DIFFERENTIAL ROLE OF POLY(A) AND INITIATION FACTORS IN THE TRANSLATION OF VIRAL AND CELLULAR mRNAS.

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We have recently demonstrated that the poly(A) moiety of short RNAs obtained from both in vitro transcription and from vaccinia virus (VV) infected cells exhibit a dissimilar effect on the in vitro translation of cellular and VV mRNAs (Bablanian et al., 1986; Su and Bablanian, 1990). We have investigated the role of poly(A), m^7GTP, and initiation factors in the mechanism of selective translation of VV mRNAs. The effect of unfractionated (termed poly(A)$_{un}$ with various chain lengths up to 3000 nucleotides) and a 150-300 nucleotide fraction of synthetic poly(A) (termed poly(A)$_{150-300}$) on the translation of HeLa cell mRNAs and early and late vaccinia virus (VV) mRNAs was studied. Both the poly(A)$_{un}$ and the poly(A)$_{150-300}$ completely inhibited the translation of HeLa cell mRNAs obtained from total cytoplasmic RNA in the nuclease-treated reticulocyte lysates. Viral mRNAs from total cytoplasmic RNA also were slightly inhibited (15-38%) by the poly(A)$_{un}$ whereas, the poly(A)$_{150-300}$ had no significant effect on their translation. The translation of oligo(dT)-cellulose selected HeLa mRNAs was as sensitive to inhibition by poly(A)$_{150-300}$ as the mRNAs found in total cytoplasmic RNA. However, the translation of oligo(dT)-cellulose selected viral mRNAs become more sensitive to the inhibitory effect of poly(A)$_{150-300}$ than the viral mRNAs found in the total cytoplasmic RNA. Both HeLa and VV mRNAs became more resistant to the poly(A)-mediated inhibition when these mRNAs were deadenylated, but, the relative resistance to inhibition by poly(A)$_{150-300}$ of deadenylated VV mRNAs was much greater than that of HeLa cell mRNAs. The translation of VV mRNAs was significantly less inhibited than the translation of HeLa mRNAs when the cap analog, m^7GTP, was added to the cell-free system. The inhibition of HeLa cell mRNA translation by both poly(A)$_{un}$ and poly(A)$_{150-300}$ was completely restored when poly(A) binding protein (PAB) was added to the cell-free translational system. The addition of initiation factor 4A (eIF-4A) did not restore translation when unfractionated poly(A) was used to inhibit trans-

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THIRD SESSION

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CONTROL OF INITIATION IN ADENOVIRUS INFECTED CELLS

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The initiation of protein synthesis in adenovirus infected cells is regulated during the late phase in two ways. The overall translation rate is maintained by a small viral RNA, VA RNA_I, which prevents the phosphorylation of initiation factor eIF-2 by a double-stranded RNA-activated protein kinase, DAI. DAI is induced by interferon and plays an important role in controlling protein synthesis in infected cells and in establishing the anti-viral state. Many viruses have developed mechanisms to counter and even exploit the enzyme. DAI is activated by double-stranded RNA (dsRNA) produced during viral infection in a reaction that involves enzyme autophosphorylation. Once activated, DAI phosphorylates the initiation factor eIF-2, preventing it from being recycled by GEF (the guanosine nucleotide exchange factor, eIF-2B) and bringing initiation to a halt. The activation of DAI is tightly regulated: it follows second-order kinetics, suggesting that two molecules of DAI are involved, and it exhibits a rigorous and complex dependence on RNA. Low concentrations of dsRNA activate, while high concentrations of dsRNA prevent activation. Small effector RNAs, including adenovirus VA RNA, the Epstein Barr virus RNAs (EBERs), and a short RNA transcribed from the HIV-1 leader region, also prevent activation of DAI.

In addition, the relative efficiency of translation of host cell and viral mRNA populations is regulated in the infected cell during the late phase such that viral mRNAs are selectively utilized. Three viral elements have been implicated in this process: (1) the 5' leader present on most late viral mRNAs which appears to render viral mRNA translation less dependent on initiation factor eIF-4E; (2) the late protein, 100K, which has mRNA-binding activity; and (3) VA RNA which interacts with DAI. The mechanisms underlying these quantitative and qualitative controls of translation rate will be discussed.

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SUPPRESSION OF CELLULAR PROTEIN SYNTHESIS BY ADENOVIRUS INVOLVES INHIBITION OF CAP-BINDING PROTEIN COMPLEX ACTIVITY

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Infection of cells with adenovirus results in a profound suppression of cellular protein synthesis which occurs with the onset of the late phase of the infectious cycle. We have previously demonstrated that a common 5' noncoding region found on viral late mRNAs called the tripatite leader confers the ability to translate with little if any requirement for cap binding protein (CBP) complex. We now show that adenovirus inhibition of cellular translation correlates with the quantitative under-phosphorylation and subsequent inhibition of cap binding protein (CBP), a component of CBP complex. The dephosphorylation of CBP is shown to occur with kinetics consistent with the inhibition of cellular translation, and to involve approximately 90% of CBP. In previous experiments we showed that the drug 2-aminopurine could largely prevent the inhibition of cellular protein synthesis by adenovirus. Here we show that 2-aminopurine also prevents much of the dephosphorylation of CBP at late times during viral infection. Finally, we demonstrated that late adenovirus mRNAs translate efficienty in mitotically arrested cells in which host protein synthesis has been inhibited by dephosphorylation of CBP.

In more recent experiments we have addressed whether the reduced phosphorylation of CBP during adenovirus infection results from enhanced CBP phosphatase activity or a block in CBP kinase function. Pulse-chase analysis of the half-life of CBP phosphate groups in uninfected and late Ad infected cells indicates that phosphate turnover occurs at relatively the same rate, with a half-life of about 20-30 minutes. Treatment of cells with the potent phosphatase inhibitor okadaic acid results in a rapid and extensive accumulation of phosphorylated CBP only in uninfected cells. Thus, both experimental approaches indicate that the activity of the CBP kinase is blocked in adenovirus infected cells. Additional experiments including those with in vitro extracts that replicate the block to CBP kinase activity observed in vivo will be presented.

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Interferon-induced and double-stranded RNA activated proteins as enzymes in regulating cellular protein synthesis

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Treatment of cells with interferon results in the induction of two double-stranded RNA activated enzymes : a specific protein kinase and 2'-5' oligoadenylate (2-5A) synthetases. The protein kinase once phosphorylated acquires the capacity to phosphorylate eIF2 and thus mediating inhibition of initiation of protein synthesis. The 2-5A synthetases form 2-5A molecules which activate a latent riboendonuclease responsible of RNA (polyadenylated and ribosomal) degradation. Accordingly these enzymes play key roles in two independent pathways by which cellular protein synthesis might be blocked.

The p68 kinase.

The double-stranded (ds)RNA activated protein kinase from human cells is a 68 kDa protein (p68 kinase) which is induced by interferon. On activation by dsRNA in the presence of ATP, the kinase becomes autophosphorylated and can catalyze the phosphorylation of the alpha subunit of eIF2. We have recently isolated several related cDNAs from which can be deduced the full length p68 kinase sequence. All of the cDNAs identify a 2.5 kb RNA which is strongly induced by interferon. A dose-dependent increase in the steady state levels of this RNA occurs within a few hours after addition of interferon to lymphobastoid (Daudi) cell cultures. Interferon-induced transcription of the 2.5kb RNA assayed in isolated nuclei is not inhibited by cycloheximide and is thus a primary response. The deduced amino-acid sequence of the p68 kinase predicts a protein of 550 amino acids

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containing all of the conserved domains specific for members of the protein kinase family including the catalytic domain characteristic of serine/threonine kinases.

In vitro translation of a reconstructed full-length p68 kinase cDNA yields a protein of 68 kDa which binds dsRNA, is recongnized by a monoclonal antibody raised against the native p68 kinase and is autophosphorylated. The full length p68 kinase cDNA was inserted in the pcDNA1/neo eukaryotic expression vector under the control of the CMV promoter and used to transfect murine NIH/3T3 cells. Stable transfectants were isolated and were shown to express the human dsRNA activated p68 kinase as demonstrated by in vitro phosphorylation.

The 2-5A synthetases.

The existence of 40-46, 69 and 100 kDa forms of 2',5'oligoadenylate (2-5A) synthetase has been established in interferon-treated human cells. Immunoenzymatic staining analysis of interferon-treated cells indicated that a proportion of p69 is concentrated around the nuclei and the rest is distributed in a specific pattern in the cytoplasm whereas p100 is found in a diffuse state in the cytoplasm. In accord with its association to cell membranes, p69 is myristilated. Although both p69 and p100 are induced by alpha or beta interferon, there are significant differences in the interferon dose-response and the kinetics of synthesis of each protein. Furthermore, treatment of some human cell lines (HeLa, Hep-2 and U937) with gamma interferon results in a synergistic induction of p69 but not p100. These observations indicate that there must be different mechanisms for the signalling pathways required for the induction of p69 and p100.

Polyclonal antibodies specific for p69 were used for immuno-screening of a random-primed cDNA library lambda gt11. One of the isolated cDNAs (436bp) was then used as a probe to screen an oligo-dt primed cDNA library constructed in lambda gt10. Three cDNAs of 2.4, 2.8 and 4.5 kb were isolated. The open reading frames of cDNAs 2.4 and 4.5 are identical whereas that of cDNA 2.8 has some 30 amino-acids more at the 3'terminus. The amino-acid sequence of the 40-46 kDa 2-5A

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synthetase shows 65 % homology to the deduced amino-acid sequence encoded by these cDNAs. In Northern blot assay, these cDNAs hybridize to several interferon-induced mRNAs of 4.5, 3.9, 3.3 and 2.8 kb in size. The three isolated cDNAs should express proteins related to p69 since they express the epitope reacting with the polyclonal antibody specific for this form of 2-5A synthetase.

Conclusion : Function of the p68 kinase and 2-5A synthetase.

The research carried out by different laboratories since 15 years, has pointed out that the 2-5A synthetase and the protein kinase play key roles in systems which mediate inhibition of protein synthesis. There is no doubt that the 2-5A system plays an important role in mediating resistance to virus infection. In addition, there has been much speculation about its role in the control of cell growth, proliferation and differentiation. The demonstration that different forms of 2-5A synthetase exist, and which manifest different properties, is further evidence supporting the investigation of alternative roles, for the different forms of 2-5A synthetase, and their product molecules. Other functions have also been attributed to these enzymes. For example, the 2-5A synthetase because of its 2'-5' nucleotidyl transferase activity has been suggested to play a role in the mechanism of splicing of precursor RNA transcripts. At present, the physiological function of the p68 kinase remains to be shown clearly although it is most probable that the p68 kinase is involved in translational control by mediating phosphorylation of eIF2.

In view of the different possible functions of these enzymes, the controlled expression of cDNAs encoding the p68 kinase and the different forms of 2-5A synthetase should be useful in determining their role in control and IFN-treated cells.

Aknowledgments

This work was carried out in collaboration with different members of my group : Eliane Meurs, Isabelle Marié, Julien Galabru, Josette Svab and Nadine Robert. Most of the work on characterization and cloning of the protein kinase was carried out by J. Galabru and I.

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Meurs, while the work on the cloning of p69 (2-5A synthetase) was carried out by I. Marié. Collaborations were carried out with laboratories headed by Bryan Williams (Toronto), Michael Katze (Seattle) and Nahum Sonenberg (Montreal).

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REGULATION OF THE INTERFERON-INDUCED dsRNA ACTIVATED PROTEIN KINASE IN ANIMAL VIRUS-INFECTED CELLS

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Animal viruses must downregulate activity of the interferon-induced protein kinase (referred to as P68) to avoid the kinase's negative effects on protein synthesis initiation. Recent data on the regulation of the protein kinase in cells infected by influenza virus and poliovirus will be presented. We have previously found that influenza virus blocks kinase activity by activating a cellular inhibitor of P68. Preliminary data suggests this activation occurs by a complex series of events which will be described. We have prepared peptide antibody against this inhibitor which has been purified to near homogeneity and has a molecular weight of 58,000 daltons. Analysis of the reactivity of this antibody with both mock and virus-infected extracts will be presented. We will also describe the molecular mechanisms of action of this cellular repressor and studies on our progress in cloning the 58kD protein. In contrast to influenza, poliovirus downregulates P68 by inducing its degradation during infection. To determine the molecular mechanisms of degradation, an in vitro assay has been developed. This has allowed us both to biochemically characterize the protease activity and begin to purify the responsible protease based on its functional activity. These data as well as studies mapping the kinase's protease sensitive sites will be presented.

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Factor-dependent yeast extracts for studying initiation of protein synthesis

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We are studying the functional properties of protein synthesis initiation factors like eIF-4E, the cap binding factor, or eIF-4A, which is required for the unwinding of secondary structures at the 5'-end of mRNAs, in the yeast *S.cerevisiae*.

To obtain more information about the mechanistic details of translation initiation we have followed a new approach, which basically consists in obtaining conditionally lethal mutants which under certain conditions produce a defective version of the factor or do not efficiently express it.
Extracts of these cell lines (pretreated at the non permissive conditions) require the addition of exogenuous wild-type factor for efficient translation.
These systems allow us to approach questions concerning
- the structural features of these initiation factors important for proper function;
- the factor dependence of particular mRNAs such as
  a.) mRNAs with secondary structure at the 5' end
  b.) cap-dependent translation of (especially bi- or polycistronic) mRNAs.

Two different strategies for obtaining factor-dependent crude extracts and the translational pecularities of specific mRNAs will be presented and discussed.

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Initiation of translation within cells directed by the 5'non-coding region of foot-and-mouth disease virus.

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Foot-and-mouth disease virus (FMDV) is a picornavirus but differs from the enteroviruses (eg poliovirus) and the cardioviruses (e.g encephalomyocarditis virus (EMCV) in significant ways. These are that the polyprotein commences at two distinct positions to produce two forms of the Leader (L) protein which self cleave from the capsid precursor and inhibit cap-dependent translation at least in part by initating the cleavage of the p220 cap-binding complex protein.

A region of about 435 bases from the 5'non-coding region of FMDV is sufficient to direct efficient internal initiation of protein synthesis (Belsham & Brangwyn (1990) J Virol 64 5389-5395) within cells and is termed the IRES. This region is immediately upstream from the first of the two AUG initiation codons which are used in vitro and in vivo and are conserved in all seven serotypes of FMDV. The initiation codons are at nt 807 and nt 891 and represent the 8th and 9th AUG codons downstream from the polyC tract. The FMDV L protein inhibits the translation of reporter genes unless they are preceded by the IRES, this can be shown in both mono-cistronic and bi-cistronic mRNAs within a transient expression system in vTF7-3 (a recombinant vaccinia virus expressing the T7 RNA polymerase) infected BSC40 cells. We have now explored whether the IRES modifies the use of the two initation codons when assayed in the presence or absence of the L protein. It has been shown previously (Kaminski et al EMBO J) that the EMCV IRES (which is structurally analagous to that of FMDV) precisely directs initiation of translation to AUG 11 while ignoring AUG 10 only 7 nt upstream. We have constructed open reading frames containing the FMDV sequences encoding the two start sites linked to a portion of the CAT gene (maintaining the open reading frame). When assayed alone this cassette produces two fusion protein products (LCAT), reflecting initiation at the two sites, with similar efficiencies. The same pattern of products is also observed when the cassette is preceded by the IRES. When a plasmid encoding L is also introduced into the cells, synthesis of the LCAT products is greatly inhibited from the cassette lacking the IRES but is maintained at a similar level for each product from the construct containing this sequence. We conclude surprisingly that the selection between initiation at nt 807 or nt 891 is independent of the presence of the IRES and when cap-dependent translation is inhibited the IRES dependent initiation also occurs similarly at each site.

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Characterization of the Protease Responsible for Degradation of the Interferon-induced Protein Kinase P68 During Poliovirus Infection. Tracy Black, Leonard Meuse, Michael Katze.

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We have been investigating the regulation of the interferon-induced, double-stranded RNA activated protein kinase P68 in poliovirus-infected cells. We have previously shown that P68 was both highly autophosphorylated and activated during poliovirus infection. In accordance with these results, phosphorylation of endogenous eIF-2α also increased in poliovirus-infected cells. When physical levels of the protein kinase were measured, we unexpectedly found that the P68 protein was dramatically decreased in poliovirus-infected cells. Subsequently, pulse chase analysis revealed that P68 was significantly degraded during infection. We developed an *in vitro* assay for P68 degradation using cell extracts. This assay corroborated our *in vivo* observation and allowed us to begin to define the mechanism of P68 degradation during poliovirus infection. Characterization experiments, using the *in vitro* assay, have shown that the P68 protease is inhibited by EDTA and restored by subsequent treatment with divalent cations. In addition, pretreatment of poliovirus-infected extracts with either trypsin or ribonuclease abolished protease activity. Using a series of truncated or deleted *in vitro* expressed P68 proteins as a substrate for degradation, we are examining possible protease sensitive sites. Finally, we have begun purification of the protease from poliovirus-infected extracts taking advantage of its highly insoluble nature. The data suggests that the protease responsible for P68 degradation during poliovirus infection is a large molecular weight complex containing an RNA component. Thus far, it appears that poliovirus proteases 2A and 3C are not directly responsible for P68 degradation. Further experiments to determine the components of this complex and its mechanism of degradation are in progress.

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Cloning of the cDNA of the Heme-Regulated Eukaryotic Initiation Factor eIF-2α Kinase of Rabbit Reticulocytes: Homology to Yeast GCN2 Protein Kinase and Human dsRNA-Dependent eIF-2α Kinase. Jane-Jane Chen,* Mark S. Throop,* Lee Gehrke,*+ Irene Kuo*, Jayanta K. Pal*, Michael Brodsky* and Irving M. London*++. *Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA 02139; +Department of Anatomy and Cellular Biology, Harvard Medical School, Boston, MA 02115; ++Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

Protein synthesis in intact reticulocytes and their lysates is dependent on the availability of heme. In heme-deficiency, protein synthesis is inhibited at the level of initiation due to the activation of a heme-regulated inhibitor (HRI), also called heme-controlled repressor, HCR. HRI is a cAMP-independent protein kinase which specifically phosphorylates the α subunit of the eukaryotic initiation factor 2 (eIF-2α). Phosphorylation of eIF-2α in reticulocyte lysates results in the binding and sequestration of reversing factor (RF), also designated as guanine nucleotide exchange factor (GEF) or eIF-2B, in an RF·eIF-2(αP) complex. Since RF is required for the exchange of GTP for GDP in the recycling of eIF-2 and in the formation of the eIF-2·Met $tRNA_f$·GTP ternary complex, its unavailability results in the cessation of the initiation of protein synthesis.

We have cloned the cDNA of HRI of rabbit reticulocytes. In vitro translation of mRNA transcribed from the HRI cDNA yields a 90 kDa polypeptide which exhibits eIF-2α kinase activity and is recognized by a monoclonal antibody directed against authentic HRI. The open reading frame sequence of the HRI cDNA contains all eleven catalytic domains of protein kinases with consensus sequences of serine/threonine protein kinases in conserved catalytic domains VI and VIII. The HRI cDNA also contains an insert of approximately 140 amino acids between catalytic domains V and VI. The HRI cDNA coding sequence has extensive homology to GCN2 protein kinase of S. cerevisiae and to human double stranded RNA-dependent eIF-2α kinase. This observation raises the possibility that GCN2 protein kinase may be an eIF-2α kinase in yeast. In addition, HRI has significant homology to three protein kinases (Nim A, Wee1 and CDC2) which are involved in the regulation of the cell cycle.

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Non-AUG Start Codons on the P/C mRNA of Paramyxoviruses Function in the Modulation of Downstream Expression.

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The number of non-AUG start sites identified in eukaryotic systems has been steadily increasing. Initially they were observed in animal viruses, but recently a number of cellular examples have appeared in the literature. The apparent "leakiness" of these non-AUG starts opens a number of possibilities to the cell:

1. the non-AUG start site, generally positioned upstream of the first AUG, can allow access to another reading frame whilst still permiting initiation at the downsteam site.
2. it can allow the synthesis of a N-terminally elongated form of the downsteam protein product which may have a different cellular function.
3. modulation of the "leakiness" of the non-AUG start could also serve as a form of translational control.

Several years ago we described the use of an ACG (position 81 on the mRNA) start site in the P/C mRNA of Sendai virus to initiate the C' protein. This protein is an N-terminally elongated form of the C protein initiated at the second AUG (position 114) and is positioned in the +1 reading frame relative to the first AUG (position 104) which is used to initiate the P protein. Recently, we have noticed non-AUG initiation sites in other members of this virus family. We have identified a GUG start site in hPIV1 (ACAGUGG) which, like Sendai virus, is used to synthesis a C' protein. More interestingly, preliminary data indicate that in hPIV3 there is also an upstream GUG start codon (AGAGUGG) but in the P ORF. This GUG is used to initiate a N-terminally elongated form of the P protein (P'). There appears to be no C' protein in hPIV3. Based on this, we propose that the upstream non-AUG start sites are probably involved in modulating expression at the downstream AUGs. Since the P protein is an essential component of the viral polymerase this, translational control may have important effects on viral replication.

We are currently examing this control using mutated constructs of the hPIV1, hPIV3 and Sendai P/C genes expressed *in-vivo*.

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Possible Role of the 5'Poly(A) Leader of Late Vaccinia Virus mRNAs in Translation

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All late but only a few early and intermediate Vaccinia Virus mRNAs studied so far have a capped poly(A) stretch of 15 to 35 nt as a 5' untranslated leader which is not encoded by the viral genome. The poly(A) leader might play a role in regulation of gene expression on the translational level. Different possibilities can be envisaged:

1) Due to extensive symmetrical transcription, together with the absence of a precise transcription termination late in infection, late mRNAs may exist in a double stranded conformation with the exception of the poly(A) head. The poly(A) head may now be long enough to enable the assembly of the 48S initiation complex thereby rendering the mRNA translatable even in the presence of its antisense RNA.

2) One aspect of the poly(A) head is that it represents a 5' non coding region without secondary structures. The assembly of the 48S complex is strongly inhibited by secondary structures. Weak hairpins however can be melted by a helicase activity associated to the factor eIF-4F. This function might be inactivated in the course of infection thus rendering the translation more dependent on mRNAs without secondary structures at the 5' end.

Experiments will be presented addressing these possibilities.

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INTERACTION OF HELA PROTEINS WITH SPECIFIC DOMAINS OF POLIOVIRUS RNA 5' NON-CLONING REGION

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UV crosslinking was used to detect HeLa cell proteins that interacted stably with the 5' noncoding region (NCR) of poliovirus RNA. At least four major protein-RNA complexes were identified, three of which were shown by RNA competition analysis to bind specifically to defined domains within the 5'NCR. Protein A (52 kDa) crosslinked to RNA sequences and/or structures located between nucleotides 457-626; proteins B (48kDa) and C (38kDa) to nucleotides 286-456. In order to determine whether these protein binding domains are important for the virus-specific processes of internal ribosome binding and protein synthesis initiation, translation competition assays were performed in rabbit reticulocyte lysates supplemented with HeLa cell extract. The results revealed two functional domains in the poliovirus 5'NCR. One (nts 457-626) binds general translation factors that are present in reticulocyte lysates as well as in HeLa cell extracts. Another (nts 286-456) interacts with proteins that are specific for poliovirus translation, and which are present in HeLa cells but not in significant amounts in rabbit reticulocyte lysates.

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REGULATION OF HEME-CONTROLLED eIF-2α KINASE OF RETICULOCYTE LYSATES

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We have obtained highly purified preparations of the heme-controlled eukaryotic initiation factor 2α subunit (eIF-2α) kinase (HCI) from rabbit reticulocyte lysates containing six different polypeptides. One of these is a 87-kDa (p87) phosphopeptide which appears to show an autokinase activity. The controlled digestion with trypsin of HCI preparations leads to the suggestion that phosphorylation of p87 is not needed for kinase activity and, furthermore, that another 92-kDa polypeptide could be the kinase catalytic subunit. In agreement with this, monoclonal antibodies directed against p87, that also recognize a 90-kDa polypeptide, do not interfere with eIF-2α kinase activity. Upon incubation of the HCI preparation with hemin (5-10 uM), the eIF-2α kinase is converted into an inactive form and appears to become associated with related peptides forming high molecular weight complexes which can be reversibly activated by 2-mercaptoethanol. The maintenance of the integrity of the porphyrin ring is absolutely required for kinase inactivation and although the presence of metal ion is not essential, the iron and cobalt metalloporphyrins are more effective than protoporphyrin IX. The formation of the inactive form of HCI by hemin is prevented by either N-ethylmaleimide, monoclonal antibodies directed against p87, or phosphorylation of p87. The data strongly suggest that hemin regulates eIF-2α kinase activity by promoting formation of the inactive dimer HCI•p87 via disulfide bonds and direct binding of hemin. A model for HCI regulation will be discussed.

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Transactivation of translation by the TAV protein of cauliflower mosaic virus.

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The 35S RNA of cauliflower mosaic virus (CaMV), a plant pararetrovirus, serves as both a template for reverse transcription and as a polycistronic mRNA. The mRNA has the coding potential for all the viral proteins and is the only mRNA for ORFs VII, I, II, III, and IV. CaMV was found to encode a transactivator (TAV) which enhances the expression of the downstream ORF in a bicistronic mRNA in both host and non-host plant cells. Evidence indicates that TAV is functioning at the posttranscriptional level by a novel mechanism of transactivation which is different from the transactivators encoded by human immunodeficiency virus type I.

Transactivation by TAV was examined by constructing a bicistronic mRNA that contained the β-glucuronidase (GUS) gene as the upstream ORF and chloramphenicol acetyltransferase (CAT) gene as the downstream ORF. The downstream ORF was expressed only under specific conditions that suggested two pathways for ribosomes to reach this downstream ORF. 1). Reinitiation: The downstream CAT ORF is translated by ribosomes that also translated the upstream GUS ORF or at least migrated through the upstream coding region. 2). Ribosome shunt: The downstream CAT ORF is translated by ribosomes that are transferred directly from a region close to the 5' end of the bicistronic mRNA to a region closely upstream of the CAT ORF.

Animal viruses have provided model systems in which to examine the mechanisms of reinitiation of translation of ORFs in bicistronic mRNAs. However, a mechanism similar to transactivation of translation by CaMV has not been identified in the animal system. The question arises as to whether transactivation of translation by TAV is restricted to plants or can occur in other systems. To examine the ability of TAV to function in a heterologous system, transactivation is being studied by expressing TAV in mammalian cells and examining its function.

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Search of poliovirus proteins responsible for membrane modifications.

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Poliovirus infection leads to drastic changes on host-cell membranes. These alterations include changes in phospholipase C and A2 activities, increase in membrane permeability to cations and low M.W. metabolites, enhanced phospholipid synthesis and induction of proliferation of membrane vesicles. These changes in phospholipid turnover and membrane proliferation induced by virus infection are required for genome replication of some animal viruses, including poliovirus. On the other hand, modification of membrane permeability may be responsible for various harmful effects on host cells induced by virus infection, such as the cytopathic effect and cell killing, modification of macromolecular synthesis, changes in the membrane potential, etc. In spite of the importance of these effects for the virus life cycle , little is still known about the mechanisms , or the poliovirus components involved .

As a first step towards the elucidation of the poliovirus proteins responsible for these membrane alterations we have cloned the non-structural proteins of poliovirus into an inducible prokaryotic system (Rosenberg et al, Gene 56: 125-135) which expresses heterologous genes from a T7 promoter. The T7 RNA polymerase is supplied from the bacterial chromosome in an IPTG -inducible manner. Constitutive expression of the T7 lysozyme reduces the basal level of active T7 RNA polymerase, allowing the cloning and expression of highly toxic genes. Recombinant bacteria that express individual non-structural poliovirus proteins or combinations of some of them have been isolated. Some bacterial clones contain the cistrons encoding polypeptides 2B, 2C, 3A or 3D, which have been mutagenized to include the start and stop codons for translation. Other clones contain 2C3A , 2C3AB ,or polyproteins including the 2A ,or 3C proteases and their flanking sequences. All the proteins are highly expressed upon induction with IPTG and rifampycin. Curiously enough, when some of these genes (3AB and the 2B) are induced , the proteins made are highly toxic and lyse the bacteria within a few minutes. At least in the case of the 3AB protein, lysis is accompanied by drastic permeability changes of the inner bacterial membrane.

To get insight into the role played by poliovirus proteins in the modification of the host-cell membrane in mammalian cells the cistrons for the 3A, 2B and 3AB proteins have been cloned in eukaryotic expression vectors. These poliovirus proteins will be expressed in both transient or inducible systems and the effects on the cell membrane in eukaryotic cells will be analyzed.

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TRANSLATIONAL COMPETITION BETWEEN VIRAL AND CELLULAR mRNAs GOVERNS THE PROGRESSION OF THE INFECTION IN AVIAN REOVIRUS S1133-INFECTED MOUSE L CELLS

Mallo, Moisés; Martínez-Costas, José Manuel & Benavente, Javier

We have previously found that the avian reovirus S1133 can replicate in mouse L cells at physiological or moderately acid pH. In order to get further insight into the molecular mechanisms that govern viral replication in this system, we have now studied the effects that the multiplicity of infection (m.o.i.) and the inhibition of cellular mRNA synthesis have on viral macromolecular synthesis and viral growth. We have found that viral yield and the number of actively infected cells were directly correlated with m.o.i. We also found that the addition of 0,5 μg/ml of actinomycin D (ActD) at the beginning of the infection resulted in an increase in viral protein synthesis, in the number of actively infected cells and in viral growth at late times of infection. The drug did not elicit this effects by facilitating reinfection by newly formed progeny virions but by triggering the replication of cytoplasmic viral particles in cells in which the virus would not replicate in the absence of ActD.

Together, these results suggest a model for S1133-L cell interaction in which translational competition between cellular and viral mRNAs determines the fate of the infection in each particular cell. According to this model the ratio between viral and cellular mRNAs must exceed a certain threshold for the infection to progress. Cells in which the ratio is smaller than the threshold will not be able to synthesize enough viral proteins for the infection to succeed, but in cells in which the viral mRNA levels are sufficient for the threshold ratio to be exceeded, viral progeny particles will form which will support the progression of the infection. Assuming that the number of active virions that penetrate host cells varies from cell to cell and that the amount of viral transcripts in each cell is proportional to the number of its active cytoplasmic particles, this model predicts that the higher the m.o.i. the more cells will have a viral to cellular mRNA ratio allowing active infection; this is indeed what is observed. This model can also explain the stimulatory effect of ActD found at late times of infection. At the ActD concentration used host mRNA synthesis is blocked without any effect on viral transcription; the amount of cellular transcripts in ActD-treated cells therefore progressively decreases, which results in more cells with an above-threshold viral to cellular mRNA ratio and hence, in an increase in the number of actively infected cells at late infection times.

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CLONING OF POLIOVIRUS 2A PROTEASE IN EUKARYOTIC EXPRESSION VECTORS.

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When Poliovirus infects Hela cells it causes a drastic inhibition of cellular protein synthesis early during infection . The 2A protein, one of the two proteases encoded by poliovirus, is indirectly involved in the cleveage of a p220 polypeptide that forms part of the Cap Binding Complex. Therefore , it has been suspected that the 2A protease might be responsible for the inhibition of translation of cellular mRNAs induced by poliovirus infection. To test this possibility, we made constructions of the poliovirus genome containing a number of internal deletions . In transient expression experiments we analyzed the effect of transfection of COS cells with these constructions on the shut-off of cellular protein synthesis. In addition, the effects of these deletion mutants on the expression of a reporter gene (Luciferase) were examined. A mutant containing a deletion that spans from 866 to 3083 inhibits luciferase expression , whereas a mutant with a deletion between 3235 to 3925 (2A-B proteins of poliovirus) does not inhibit the luciferase activity. This effect also correlates with a lack of RNA replication in the last mutant. Thus, it is not clear at present if the inhibition on luciferase activity is due to the lack of 2A or to the absence of RNA replication in that construct.

To examine the relationship between 2A and the shut-off of translation of capped mRNAs, we are carrying out studies using two inducible expression systems for eucariotic cells. A construct was made containing the 2A protein of poliovirus located after the 5´ leader sequence of poliovirus under the promoter of T7 RNA polymerase. COS cells were transfected and clones were selected that expressed 2A transcripts upon infection with a recombinant vaccinnia virus that encodes the T7 RNA polymerase. The other system used takes advantage of a rabbit cell line that constitutively expresses the Lac repressor and the T3 RNA polymerase. In that system the construction leader-2A is under the T3 promoter followed by the Lac operator. Synthesis of 2A in this construct can be induced by the addition of IPTG. The level of expression of 2A and the effects of 2A on translation in these two systems are being analyzed.

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PCR AMPLIFICATION OF FMDV RNA FRAGMENTS: APPLICATION TO THE STUDY OF GENE EXPRESSION IN EUKARYOTIC CELLS AND OF FMDV GENETIC VARIABILITY.

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Foot-and-mouth disease virus (FMDV) is characterized by two important features : 1) its ability to infect persistently animals, and cells in culture; 2) the genetic heterogeneity, shared with other RNA viruses whose genomes are described as quasispecies.

Virus isolated from cell lines persistently infected differ from the parental virus in point mutations distributed all along the genome. We have cloned defined fragments such as the IRES region from both viruses to understand the role that different mutations played in the phenotypic alterations observed in the persistent virus.

To facilitate the cloning of specific fragments of the viral genome we have applied the PCR methodology to the amplification of defined regions of FMDV type C. The efficiency of amplification was at least 2.5×10^7 per initial molecule. We have been able to detect in ethidium bromide stained agarose gels DNA amplified from 4×10^3 RNA molecules.

PCR products have been cloned in pUC plasmid vectors. The inserts of pUC plasmids were transferred to eukaryotic expression vectors based on the Herpes simplex tk promoter and the firefly luciferase as reporter gene. T7 and SP6 promoters from pGEM vectors are also present in the vector.

Cotransfection of the plasmids coding for FMDV sequences with pSV2PAC, that confers puromycine resistance, resulted in constitutive expression, determined at the level of transcription, of the 3C protease gene, the 3D polymerase gene, and the 400 nucleotide fragment corresponding to the 3' end of the FMDV RNA.

Direct PCR sequencing offers the possibility of determining the genomic sequence of a virus without the need to purify large amounts of RNA. Sequences obtained by this method have been identical to those found by direct sequence of the viral RNA. Extension of this methodology to several genomic regions and to different isolates will help us in determining the appearance of new variant genomes and to better approach the important problem of virus variation in nature.

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THE SMALLER OF THE TWO LEADER PROTEINS (Lb) OF FOOT-AND-MOUTH DISEASE VIRUS INDUCES INHIBITION OF CAP-DEPENDENT TRANSLATION *IN VIVO*.

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Foot-and-mouth disease virus (FMDV) form the genus aphthovirus in the family *Picornaviridae* which also includes the enteroviruses, rhinoviruses and cardioviruses. These viruses have genomes of positive sense ssRNA which, in the case of FMDV, is approx. 8.5 kb in length. The genome is expressed as a single polyprotein which is cleaved into mature proteins. The structural protein precursor region (P1) of the entero- and rhinoviruses starts at the N terminus of the polyprotein but in the cardio- and aphthoviruses P1 is preceded by a short leader protein (L).

The aphthoviruses are further characterized by the presence of two independent translation initiation AUG codons in the same reading frame and located 84 nucleotides apart. The presence of these two functional initiation codons is a highly conserved feature in viruses of all seven serotypes of the aphthoviruses. Both of these codons initiate protein synthesis *in vivo* and *in vitro* to produce alternative forms of the leader protein (Strebel and Beck, 1986, J.Virol. 58,893; Sangar et al., 1988, J.gen.Virol. 69, 2327). The larger of these (Lab) is initiated from the first functional AUG and contains all the sequence of the shorter leader (Lb) plus an N-terminal extension of 28 amino acids.

Multiples activities have been assigned to the L protein. It cleaves itself (in cis or trans) from the amino-terminus of the P1 protein. (Strebel and Beck, 1986, J.Virol. 58, 893). It is also responsible for initiating the cleavage of the cap-binding complex component p220 (directly or indirectly)(Devaney et al., 1988, J.Virol. 62,4407) and it is probable that a second effect on protein synthesis occurs since inhibition of cap-dependent mRNA translation is almost complete (Belsham and Brangwyn, 1990, J.Virol. 64, 5389).

We have constructed plasmids which encode Lb alone, Lab alone, or the two together and examined the ability of the proteins produced to cleave in trans the L/P1 junction, to inhibit the translation of a reporter gene and to cleave p220 within cells.

Expression of proteins from these plasmids was assayed using the transient expression assay developed by Fuerst et al. (1986, P.N.A.S. 83, 8122) as described previously (Belsham et al., 1990, Virology 176, 254). The cleavage of the L/P1 junction in trans was assessed by the removal of the residual L protein sequences from the amino-terminus of 1AB obtained by expression of the CA103 cassette (Belsham et al., 1990, Virology 176, 254). The inhibition of cap-dependent mRNA translation was monitored by cotransfection with a bicistronic plasmid encoding the GUS and CAT genes, the latter preceded by the IRES of FMDV. The cleavage of p220 was studied by Western blot analysis using a monoclonal antibody which was a generous gift from Dr. D. Etchison.

The activities of the plasmid encoding Lb are indistinguishable from that encoding both forms of the leader protein. We observe processing *in trans* of the L/P1 junction, inhibition of GUS activity and cleavage of p220. Thus the smaller of the two proteins has all the activities attributed to the L protein. The activities of Lab are under investigation.

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ADENOVIRUS VA RNA - MUTAGENIC AND PHYLOGENETIC ANALYSIS OF STRUCTURE AND FUNCTION.

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VA RNA_I is a key viral product which enables adenovirus to resist the interferon-induced cellular antiviral defense mechanism. VA RNA is a structured 160 nt polIII transcript, produced abundantly in cells at late times of infection. It competes with dsRNA for binding to DAI, the dsRNA-activated inhibitor of protein synthesis. This kinase is activated by autophosphorylation and phosphorylates the initiation factor eIF-2 thus causing cessation of protein synthesis. Based on nuclease sensitivity analysis a secondary structure model was proposed for VA RNA_I consisting of three main regions: an apical stem-loop, central domain (CD) and terminal stem. To study the features of VA RNA_I that are important for function we have made deletion and substitution mutants. The RNAs were tested for function *in vivo* and *in vitro* and their structure was analyzed in solution. We have concluded that an apical stem structure and the CD are required for function. The efficient binding of VA RNA_I to DAI depends on a minimal stem structure containing at least 6 base pairs; the size and sequence of the loop and the sequence of the stem can vary. Large substitutions that prevent the formation of the apical stem abolish VA function which can be restored by base compensating mutations that reform the stem. Small substitutions that allow the formation of a short stem-loop structure impair function only slightly even though the base of the stem is perturbed. However, a "mini" VA RNA_I mutant that retains only a short stem does not function *in vitro*, suggesting that the distance of the stem-loop from the CD is important for function.

Structure-function relationships within the CD were investigated by introducing foreign sequences into a 20 nucleotide region. Some sequences abolish or impair the function of the mutants in kinase inhibition assays while other sequences are neutral or even improve the inhibition function as compared to wild-type. Nuclease sensitivity analysis showed that changes in the RNase protection pattern are confined to the CD and do not affect the structure of the apical stem-loop or the terminal stem. Correlation of the structural changes in the CD with the function of the respective mutant RNAs suggests that an alternative conformation may be important for function and implies the existence of tertiary interactions between the CD and a neighboring interior loop. Phylogenetic comparison with the VA RNAs of other adenovirus serotypes shows that the secondary structures are broadly similar but differ in detail, suggesting that a common tertiary structure is the key to VA RNA function.

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LACK OF CORRELATION BETWEEN CLEAVAGE OF p220 AND POLIOVIRUS-INDUCED SHUT-OFF OF CELLULAR PROTEIN SYNTHESIS

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After poliovirus infection a few cellular proteins become degraded (Urzainqui & Carrasco (1989): J. Virol 63, 4724-4735). Amongts those proteoliticaly degraded proteins, a polypeptide known as p220 that forms part of the CBP-complex has been more intensively studied . It has been suggested that cleavage of p220 leads to the selective inhibition of cellular translation of capped mRNAs. In order to investigate the relationship between the shut-off of host protein synthesis and the cleavage of p220 we carried out detailed kinetic studies of both events after poliovirus infection of HeLa cells. In agreement with previous findings (Etchison et al (1982): J. Biol. Chem. 257, 14806-14810; Bonneau & Sonenberg (1987): J. Virol. 61, 986-991) we observed that at early times of poliovirus infection when the level of cellular protein synthesis has not yet been abated (80% of uninfected control cells) intact p220 is not detected and p220 is in the form of degradation products. Aproximately one hour later host protein synthesis undergoes a drastic fall. These results suggest that the inhibitor responsible for the shut-off is generated after p220 cleavage.

To check in more detail the possibility that poliovirus-infected cells are able to synthesize cellular proteins when p220 has been degraded we made use of inhibitors of poliovirus replication: i.e. guanidine and Ro 09-0179. When cells are infected at a multiplicity of 20 pfu/cell in the presence of these agents over 70% of host translation was observed at two hours post-infecton; at that time p220 was already cleaved. Moreover, two hours later (4 h.p.i.) a significant amount of host translation was still taking place (55%), even though p220 was degraded. These results agree well with previous findings using these agents and suggest that p220 becomes degraded even when very low amounts of poliovirus proteins (2A) are made. In spite of this degradation substantial amount of cellular proteins are synthesized.

To further examine the correlation between p220 cleavage and the blockade of host protein synthesis by poliovirus we searched for conditions able to prevent the shut-off of translation. We found that when HeLa cells were infected at 37°C during half an hour and then shifted to 28°C poliovirus replication is restricted and no inhibition of cellular protein synthesis is detected. Under these conditions p220 is cleaved at a lower rate . Interestingly enough, poliovirus-infected cells were still able to synthesize cellular proteins at the same level as mock-infected cells when the temperature was raised to 37°C. Intact p220 was not detected under these conditions and was in the form of cleavage products by that time.Preliminary data suggest that the restriction of low temperature is located on poliovirus RNA replication.Further experiments will be aimed to study the proteolysis of p220 under conditions in which poliovirus does not exert a profound inhibition of host protein synthesis.

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IDENTIFICATION OF THE INITIATION CODON OF PLUM POX POTYVIRUS GENOMIC RNA

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The expression of plum pox potyvirus (PPV) genomic RNA takes place through translation of its unique long and functional open reading frame (ORF) into a large polyprotein that undergoes extensive proteolytic processing. We show that the AUG recognized as the initiation codon of the PPV ORF by *in vitro* translation systems is the one found at nucleotide position 147, in spite of the presence at position 36 of an in-phase AUG that marks the start of the ORF. Deletion of a substantial part of the PPV 5´ nontranslated region (5´-NTR), from nucleotide 19 to 108, does not impair the *in vitro* translation of PPV synthetic transcripts. By introduction of mutations that disrupt either of these two AUGs into a full-length PPV cDNA clone, it is shown that, while alteration of the first AUG does not have any effect on virus viability, growth, or symptom induction, destruction of the second renders the viral RNA noninfectious. This result indicates that the AUG employed *in vivo* is also the second. The hypothesis that this AUG could be recognized through a ribosomal internal entry mechanism has been tested *in vitro* using bicistronic transcripts in which the PPV 5´-NTR was internally placed. The second cistron of these bicistronic RNAs was translated, although at low levels, and the significance of these results in regard to either a ribosomal internal entry or a conventional scanning mechanism for PPV RNA translation initiation will be discussed.

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CHARACTERIZATION OF mRNA CAP-BINDING PROTEIN COMPLEX FROM NORMAL AND HEAT-SHOCKED Drosophila EMBRYOS

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We are studying the mRNA cap-binding proteins (CBPs) from Drosophila melanogaster embryos and their possible role on the mechanism of the translational regulation established under heat shock conditions. Previously (MCB 9, 2181) we reported that Drosophila embryos contain a 35-kDa polypeptide which is functionally equivalent to mammalian eIF-4E. This polypeptide could be also isolated as part of a salt-stable protein complex (CBP complex) by fractionating the ribosomal high-salt wash according to a similar method used to purify the mammalian eIF-4F. In addition to eIF-4E this CBP complex contains a smear of polypeptides of 140-180 kDa and several minor components. A new method, which involves a direct fractionation of whole embryo lysate through m^7GTP-Sepharose, has been recently developed to obtain free eIF-4E and CBP complex. This new complex consists of two main polypeptides, eIF-4E and one of about 200 kDa (sometimes resolved as a doublet in SDS-PAGE), and a minor component (s) of 160-170 kDa. Polyclonal antibodies against p200 reacted with this minor component and the high molecular weigth polypeptides of the CBP complex isolated from the ribosomal high-salt wash. The 35 and 200 kDa polypeptides of the CBP complex cosedimented through sucrose density gradients containing either 0.1 M or 0.5 M KCl. As previously shown for eIF-4E (MCB 9, 2181), the p200 component of the CBP complex was also required for mRNA translation in cell-free systems from Drosophila embryos. None of the polypeptides of the CBP complex were able to cross-link (α-^{32}P) dATP upon UV irradiation. Consistent with this, we did not find any RNA-dependent ATPase activity associated with the CBP complex. From these results it is tempting to suggest that Drosophila CBP complex is the counterpart of mammalian eIF-4F but lacking the eIF-4A subunit. When the purification was carried out from heat-shocked embryos a great decrease in the amount of the CBP complex was obtained, suggesting that under heat shock conditions either the formation of the CBP complex or its interaction with m^7GTP-Sepharose is impaired. This impairment may account, at least to some extent, for the mRNA discrimination established in heat-shocked Drosophila embryos. This hypothesis is also supported by two additional results: i) When added to a lysate from heat-shocked embryos, the CBP complex was able to rescue partial but specifically the translation of the bulk of endogenous normal mRNAs (or globin mRNA added exogenously) and hsp83 mRNA. ii) By using anti-eIF-4E or anti-p200 antibodies, we found that whereas translation of the bulk of the endogenous normal mRNAs (or globin mRNA added exogenously) in the lysates was dependent on both eIF-4E and p200, the heat shock mRNAs (particularly hsp70 mRNA and with the exception of hsp83 mRNA) were translated almost independently of these polypeptides.

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INVESTIGATIONS ON HIV-1 RIBOSOMAL FRAMESHIFTING IN VIVO AND IN VITRO

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Ribosomal frameshifting is essential for the expression of the catalytic proteins (Pro, RT, Int) of HIV-1. A defined sequence of the gag-pol overlapping region has been shown to be necessary for this specific process.
We have developed an expression system allowing a simple and rapid quantification of frameshifting events in vivo and in vitro. The method is based on the expression of an N-terminally extented firefly luciferase gene which depends on -1 frameshifting at the HIV-1 derived sequence, in order to be translated as a functional enzyme.
Our data show a reduced frameshift efficiency in mammalian cells in comparison to frameshifting in vitro. We found that the stem loop structure is dispensable for frameshifting, the short homopolymeric run 'UUUUUUA' within the HIV-1 sequence mediates frameshifting in vivo and in vitro. However, deletion of the stem loop leads to a reduced frameshift efficiency in vitro.
No significant tissue or species specifity could be detected. Furthermore there is no influence on the frameshift efficiency by the status of differentiation of F9 cells.
Several inhibitors of HIV-1 replication with unknown molecular mode were tested. None of them could affect frameshifting.

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CONCLUSIONS

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TRANSLATIONAL CONTROL BY ANIMAL VIRUSES:

Nahum Sonenberg and Luis Carrasco.

Animal viruses utilize an array of strategies to express their genomes and to regulate gene expression. The regulation of the translation of viral genomes plays an important part in the final outcome of the amount of each viral protein synthesized in the infected cells. In addition, translation of viral genomes usually takes place over a profound inhibition of cellular protein synthesis has been established. Therefore, animal viruses have devised strategies to interfere with the expression of cellular proteins at the translational level, without depressing the translation of their own mRNAs. The goal of the meeting held in Sigüenza (Spain) under the patronage of the Fundación Juan March was to revise and update the motifs in viral mRNAs that make them more efficient for translation in the infected cells. The different mechanisms used by animal viruses to shut-off host translation was also a major theme of interest of the meeting.

To understand how viral mRNAs are preferentially initiated as compared to host messages, a detailed account of the number and functions of the different initiation factors is required. Moreover, we ought to understand how these initiation factors are able to interact with each other and with viral and host messages. This subject was reviewed by J. Hershey and W. Merrick, who discussed the enormous progress that has been made in the past years in cloning the genes for the numerous eukaryotic initiation factors, understanding their precise function in translation, and regulation of their activity by phosphorylation and other modifications. W. Merrick described the different mechanisms of translation initiation and particularly experiments done in his lab on internal binding of ribosome in *in vitro* translation systems.

Dr. Merrick's talk served as an introduction to several presentations by V. Agol, N. Sonenberg, R. Jackson, E. Wimmer, H. Voorma and E. Ehrenfeld, who described the extensive analysis of the 5' untranslated region in picornavirus RNAs' that serve as the internal ribosome entry site (IRES) or ribosome landing pad (RLP). Several critical nucleotides and secondary

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structure motifs were identified and characterized. Furthermore, proteins that recognize the IRES were identified and are currently purified and characterized in several laboratories. A major breaktrough was reported by E. Wimmer, who found that infectious poliovirus particles can be made in the test tube upon addition of the viral mRNA and the necessary components for translation and replication of the viral RNA.

P. Sarrow reported that internal binding of ribosomes on eukaryotic mRNAs is not unique to viral mRNA, since several cellular mRNAs from mammals and Drosophila initiated translation by a similar mechanism. These findings have major implications for the understanding of the control of translation initiation during the cell cycle, in development and differentiation.

Considerable interest has been generated by the presentations on the mechanisms of the shut-off of host protein synthesis during picornavirus infection. E. Ehrenfeld and D. Etchison described the importance of eIF-3 associated with p220 for cap recognition and the proteolysis of p220 in poliovirus and FMDV infected cells. L. Carrasco described the importance of membrane damage and consequent changes in monovalent ion concentrations on the preferential translation of viral versus cellular mRNAs. The experiments that are now in progress in Dr. Carrasco's lab are to express the different poliovirus genes in vivo and to determine the proteins that are involved in membrane modifications.

The meeting continued with the description of the mechanisms by which other viruses regulate the translation of their own mRNAs and those of the host. A. Shatkin described an interesting mechanism employed by reovirus to regulate the translational elongation of a bicistronic mRNAs. R. Garry summaryzed the evidence that the shut-off of hust translation by Togaviruses in correlated with the ionic changes in the infected cell. M. Mathews and R. Schneider described two different mechanisms by which adenovirus shuts off host protein synthesis and prevents the host defense mechanism involving interferon to interfere with viral replication. A similar mechanism for protection by the virus from the host defense machinery was detailed by M. Clemens for Epstein-Barr virus. Yet another mechanism described by R. Bablanian involving polyA transcripts was found to operate in the shut-off of host protein synthesis caused by vaccinia virus.

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The meeting was concluded by two talks describing recent work on the cloning and mechanism of action of one of the best understood mediators of interferon action - the double-stranded RNA dependent kinase, that is a major component of the cell defense against virus infection.

In summary, the latest advances in the understanding of host-virus interactions at the cellular level have been presented. It is clear that many challenges, including the development of effective anti-viral therapy, lie ahead. Nonetheless, the remarkable knowledge that has been assembled instills hope that viral disease will be conquered in the not too distant future.

We consider that this worshop succeeded by all means to fulfil the expectations of the participants both from the scientific and the social view-points. We feel that we interpret the feeling of all the participats stating that all of us very much enjoyed the facilities and the excellent food of the Parador Nacional de Sigüenza. All this was accomplished by the great expertise and the efficacy of Andrés González and the excellent staff of the Fundación Juan March in organizing these International Workshops on Biology.

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LIST OF INVITED SPEAKERS

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Workshop on
THE REGULATION OF TRANSLATION IN ANIMAL
VIRUS-INFECTED CELLS

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THE REGULATION OF TRANSLATION IN ANIMAL VIRUS-INFECTED CELLS

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