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NOTICE OF ENTITLEMENT

I, Douglas J. Campion, of U.S. Department of Commerce, 5285 Port Royal Road, Springfield, Virginia 22161, United States of America, being authorised by the Applicant/Nominated Person in respect of Application No. 60344/90 state the following:-

The Applicant/Nominated Person has entitlement from the actual inventors as follows:

The Applicant/Nominated Person is the assignee of the actual inventors.

The Applicant/Nominated Person is entitled to rely on the application listed in the Declaration under Article 8 of the PCT as follows:

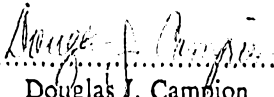
The Applicant/Nominated Person is the assignee of the basic applicants.

The basic application listed on the Declaration under Article 8 of the PCT is the application first made in a Convention country in respect of the invention.

The Applicant/Nominated Person is the depositor of the following deposits with American Type Culture Collection:

Microorganism:	pT7EMCAT
Deposit Date:	7 July 1989
Accession No.:	ATCC 68045

DATED this 9th day of March 1993


.....
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RAPID, VERSATILE AND SIMPLE SYSTEM FOR EXPRESSING GENES IN EUKARYOTIC CELLS

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(57) Claim

1. A recombinant vaccinia virus, the genome of which includes a T7 promoter followed by a cDNA copy of an untranslated region of a cardiovirus RNA that confers cap independent translation, followed by a gene encoding a protein desired to be expressed, said promoter, cDNA and the gene being so arranged that said gene is efficiently expressed in a mammalian cell infected with said recombinant virus only when said cell contains T7 RNA polymerase.
2. The recombinant virus of claim 1 wherein said untranslated region is from EMCV.
3. A recombinant plasmid comprising a T7 promoter followed by a cDNA copy of an untranslated region of a cardiovirus RNA that confers cap independent translation, followed by a gene encoding a protein desired to be expressed, said promoter, cDNA and the gene being so arranged that said gene is efficiently expressed in a mammalian cell infected with said recombinant virus only when said cell contains T7 RNA polymerase.
6. A method for synthesizing protein, comprising infecting a cell with the vaccinia virus of claim 1 or claim 2 and infecting said cell with a second vaccinia virus that expresses T7 RNA polymerase and then recovering the desired protein by suitable isolation and purification techniques.
15. A method for obtaining an enhanced amount of a desired protein, comprising employing the plasmid of claim 3 or claim 4 to construct a recombinant vector and

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then isolating the protein expressed by said vector in a suitable cellular medium by conventional isolation and purification techniques, the amount of protein thus obtained being about 500% more than the amount obtained without employing the untranslated region.

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(21) International Application Number: PCT/US90/03687 (22) International Filing Date: 3 July 1990 (03.07.90) (30) Priority data: 376,687 7 July 1989 (07.07.89) US (71) Applicant: THE UNITED STATES OF AMERICA, as represented by the Secretary, U.S. DEPARTMENT OF COMMERCE [US/US]: 5285 Port Royal Road, Springfield, VA 22161 (US). (72) Inventors: MOSS, Bernard ; 10301 Dickens Avenue, Bethesda, MD 20814 (US). FUERST, Thomas ; 604 Paradise Court, Gaithersburg, MD 20877 (US). ELROY-STEIN, Orna ; 1643 E. Jefferson, #202, Rockville, MD 20852 (US).	(74) Agents: HOLMAN, John. Clarke et al.; Fleit, Jacobson, Cohn, Price, Holman & Stern, The Jenifer Building, 400 Seventh Street, N.W., Washington, DC 20004 (US). (81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> 642657	
(54) Title: RAPID, VERSATILE AND SIMPLE SYSTEM FOR EXPRESSING GENES IN EUKARYOTIC CELLS (57) Abstract An efficient, versatile and simple expression system which confers cap-independent translation to prokaryotic RNAs in eukaryotic cells has been described. The utility of recombinant vaccinia virus for this purpose has been illustrated.		

RAPID, VERSATILE AND SIMPLE SYSTEM
FOR EXPRESSING GENES IN EUKARYOTIC CELLS

The present invention provides an improved, rapid and versatile system with a substantially high level of protein synthesis.

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SUMMARY OF THE INVENTION

An object of the present invention is to provide an efficient expression system which confers cap-independent translation to RNAs in eukaryotic cells, particularly when a prokaryotic transcription system is used in eukaryotic cells.

An additional object of the present invention is to provide a method for controlled
 10 expression of toxic genes.

Other objects and advantages will become evident from the following detailed description of the invention.

According to a first embodiment of the present invention there is provided a recombinant vaccinia virus, the genome of which includes a T7 promoter followed by a
 15 cDNA copy of an untranslated region of a cardiovirus RNA that confers cap independent translation, followed by a gene encoding a protein desired to be expressed, said promoter, cDNA and the gene being so arranged that said gene is efficiently expressed in a mammalian cell infected with said recombinant virus only when said cell contains T7 RNA polymerase.

20 According to a second embodiment of the present invention there is provided a recombinant plasmid comprising a T7 promoter followed by a cDNA copy of an untranslated region of a cardiovirus RNA that confers cap independent translation, followed by a gene encoding a protein desired to be expressed, said promoter, cDNA and the gene being so arranged that said gene is efficiently expressed in a mammalian cell
 25 infected with said recombinant virus only when said cell contains T7 RNA polymerase.

According to a third embodiment of the present invention there is provided a method for synthesizing protein, comprising infecting a cell with a vaccinia virus of the first embodiment and infecting said cell with a second vaccinia virus that expresses T7 RNA polymerase and then recovering the desired protein by suitable isolation and purification
 30 techniques.

According to a fourth embodiment of the present invention there is provided a method for synthesizing protein, which comprises transfecting a cell with the plasmid of



the second embodiment and infecting said cell with a DNA based cytoplasmic virus expressing T7 RNA polymerase, and then recovering the desired protein by suitable isolation and purification techniques.

According to a fifth embodiment of the present invention there is provided a method
5 for obtaining an enhanced amount of a desired protein, comprising employing the plasmid of the second embodiment to construct a recombinant vector and then isolating the protein expressed by said vector in a suitable cellular medium by conventional isolation and purification techniques, the amount of protein thus obtained being about 500% more than the amount obtained without employing the untranslated region.

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BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects, features and many of the attendant advantages of the invention will be



better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1 shows a schematic structure of expression cassettes in accordance with the present invention. Abbreviations: P_{T7} bacteriophage T7 ϕ 10 promoter from -23 to +1, 5'hp, nucleotides +1 to +26 downstream of the ϕ 10 promoter and capable of forming a hairpin or stem-loop structure; T_{T7}, bacteriophage T7 ϕ 10 termination sequence; EMC, nucleotides 163 to 746 of the EMCV UTR.

Figure 2 shows the results of time course study of CAT synthesis during vaccinia virus infection. CV-1 cell monolayers were infected with 20 PFU per cell of wild-type vaccinia virus (lane 9), or co-infected with vTF7-3 and vT7CAT (lanes 1, 2, 5, 6, 10, 11), or with vTF7-3 and vT7EMCAT (lanes 3, 4, 7, 8, 12, 13) at a multiplicity of 10 PFU per cell of each virus. At 2, 7 and 24 hr after infection, cells were starved for methionine for 20 min and then were pulse-labeled with [³⁵S]methionine for 30 min. Starvation and pulse labelling were done in isotonic (lanes 1, 3, 5, 7, 9, 10, 12) or hypertonic (190mM NaCl) (lanes 2, 4, 6, 8, 11, 13) conditions. Cell lysates were prepared and equal volumes (corresponding to 2x10⁵ cells) were loaded in each lane of a 10% NaDodSO₄-polyacrylamide gel. Following electrophoresis, the gel was processed for fluorography. The arrow identifies the CAT protein.

Figure 3 demonstrates the accumulation of CAT protein by 24 hr after infection. CV-1 cell monolayers were infected with 20 PFU per cell of wild-type vaccinia virus (A, lane 1; B, solid bar), or co-

infected with vTF7-3 and vT7CAT (A, lane 2; B, hatched bar), or co-infected with vTF7-3 and vT7EMCAT (a, lanes 3 and 4; B, open bar) at a multiplicity of 10 PFU per cell for each virus. At 4 hr after infection, the isotonic medium was maintained (A, lanes 1-3; B, part 1) or changed to 190 mM (hypertonic) NaCl (A, lane 4; #B part 2). At 24 hr after infection, cell lysates were prepared, 20 μ g of protein were loaded in each lane of a 10% NaDodSO₄-polyacrylamide gel and following electrophoresis the gel was stained with Coomassie brilliant blue (A) or CAT activity was determined as described in the text.

Figure 4 shows cap-independent translation of RNA containing EMCV UTR. In vitro transcription was carried out using Bam HI digested pT7CAT (lanes 1-4) or pT7EMCAT (lanes 5-8) as templates. Lanes containing unmethylated (GpppG) or methylated (m⁷GpppG) capped RNA are indicated. Translation in micrococcal-treated rabbit reticulocyte lysate was performed with RNA concentration of 8 nM with or without the presence of 0.1 mM methylated GDP (m⁷GDP) as indicated.

Figure 5 demonstrates polyribosome-association and in-vitro translation of CAT RNA made in infected cells. (A) HeLa cells were co-infected with 10 PFU/cell of vTF7-3 and 10 PFU/cell of either vT7CAT (●__●) or vT7EMCAT (0__0). After 12 hr, cytoplasmic extracts were prepared and sedimented on sucrose density gradients. Optical density at 260 nm of each fraction was measured and the position of the monosome peak is indicated. A 20 μ l sample of each fraction was immobilized on nitrocellulose using a slot blot apparatus and then hybridized to a [³²P]-CAT probe.

Autoradiograms were made and the intensities of the bands were determined by densitometry. Indicated fractions (horizontal lines) were pooled and processed for in vitro translation. (B) Samples (1 µg) of the pooled RNA were translated in a rabbit reticulocyte lysate in a final reaction volume of 12.5 µl with (+) or without (-) 0.1 mM m^7 GDP as indicated. The translation products of no RNA (lane 1), polyribosome associated T7-CAT RNA (lanes 2, 3), polyribosome-associated T7 EMCV UTR-CAT RNA (lanes 4, 5), free T7-CAT RNA (lanes 6, 7), free T7 EMCV UTR-CAT RNA (lanes 8, 9) were analyzed by NaDodSO₄-polyacrylamide gel electrophoresis and a fluorogram was made.

DETAILED DESCRIPTION OF THE INVENTION

10 The above and various other objects and advantages of the present invention are achieved by an expression system, wherein the vector is a DNA based cytoplasmic virus, RNA polymerase and promoter are from a DNA bacteriophage and the ribosome binding site is from an RNA virus. The preferred embodiment comprises a recombinant vaccinia virus constructed to include a T7 promoter, an encephalomyocarditis virus (EMCV) 15 untranslated region (UTR), and a gene for a protein desired to be expressed wherein the gene is regulated by said T7 promoter, as illustrated in Figure 1. However, it should be noted that the untranslated region that confers cap independent translation may be from sources other than EMCV including related viruses such as poliovirus and mengovirus and from unrelated viruses such as adenovirus and the like. The examples provided herein are 20 only illustrative and not limiting. For instance, following the teachings of the present invention, other



prokaryotic transcription systems such as bacteriophage SP6, T3 and GH1 could be employed for expression in eukaryotic cells.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned hereunder are incorporated herein by reference. Unless mentioned otherwise, the techniques employed or contemplated herein are standard methodologies well known to one of ordinary skill in the art. The materials, methods and examples are illustrative only and not limiting.

MATERIALS AND METHODS

Materials. Restriction endonuclease were obtained from New England Biolabs, Bethesda Research Laboratories or Boehringer Mannheim. m^7GpppG , $GpppG$, and m^7GDP were obtained from Pharmacia and T7 RNA polymerase and rabbit reticulocyte lysate from Promega Biotec.

Viruses and cells. Vaccinia virus (strain WR) was originally obtained from American Type Culture Collection. Viruses were purified as reported previously (Fuerst et al, 1986, Proc. Natl. Acad. Sci USA 83, 8122-8126). HeLa S-3 cells were grown in suspension in Eagle's minimal essential medium supplemented with 5% horse serum. CV-1 monkey kidney cells were propagated as monolayers in Dulbecco's

modified Eagle's medium containing 10% fetal bovine serum. Cells were infected as described previously (Fuerst et al, 1987, Mol. Cell Biol. 7, 2538-2544; Fuerst et al, 1989, J. Mol. Biol. 206:333-348).

- 5 Plasmid constructions. A 0.6 kilobasepair (kbp) EcoR1-Ball EMC fragment from pE5LVPO (Parks et al, 1986, J. Virol. 60, 376-384) containing nucleotides 163 to 746 of the EMCV UTR was inserted into the blunted BamH1 site of plasmid vector pAR2529 (Rosenberg et al, 1987, 10 Gene 56, 125-135 to generate pTF7.25EMC-1. The CAT coding sequence was isolated from pTF7CAT-1 (Fuerst et al, supra), as 0.8 kbp Taq^I fragment and using BamH1 linkers was inserted into the BamH1 site of pUC18 to generate the plasmid pUC18CAT-1. The 0.8 kbp BamH1 CAT 15 fragment was then isolated from pUC18CAT-1 and inserted into a unique BamH1 site of plasmid pTF7.25EMC-1 downstream of the major translation start site of EMC, to form plasmid pTF7.25EMCAT-1. This resulted in placing the CAT translation start site in frame with 20 the EMC major start signal separated by 12 amino acid residues. The coding frame of CAT was placed immediately downstream of EMC start codon by site directed mutagenesis. The sequence immediately flanking the initiating EMC ATG was changed to 5'- 25 EMC...GATAATACCATGGAG...CAT-3', which provides a unique NcoI site. An NcoI site contained within the CAT gene was removed by placing a silent mutation within the coding sequence. The mutagenized EMC CAT cassette was then isolated as a 0.82 kbp BamH1-KpnI fragment and 30 inserted into the corresponding site in plasmid pTF7.25EMCAT-1 to create pTF7.25EMCAT-20. For simplicity pTF7.25EMCAT-20 and pTF7CAT-1 will be

referred to as pT7EMCAT and pT7CAT, respectively.

A deposit of pT7EMCAT plasmid has been made at the ATCC, Rockville, Maryland on July 7, 1989 under the accession number 68045. The deposit shall be viably
5 maintained, replacing if it becomes non-viable during the life of the patent, for a period of 30 years from the date of the deposit, or for 5 years from the last date of request for a sample of the deposit, whichever is longer, and upon issuance of the patent made
10 available to the public without restriction in accordance with the provisions of the law. The Commissioner of Patents and Trademarks, upon request, shall have access to the deposit.

Vaccinia virus insertion vectors were prepared
15 by excising the complete T7 promoter-target gene-T7 terminator cassettes as BgIII fragments, blunting the ends and inserting into the blunted EcoRI site of pGS50 (Fuerst et al, supra). This resulted in flanking the target gene cassettes with vaccinia virus thymidine
20 kinase (TK) sequences used for homologous recombination. Recombination into the genome of vaccinia virus and the isolation of recombinant virus was carried out as described by Mackett et al, 1984, J. Virol. 49:857-864.

25 In vitro transcription. Plasmids were digested with BamHI to release the T7 transcription unit as a linear DNA fragment. Transcription was carried out as recommended by Promega Biotec except that the GTP concentration was 0.05 mM and m⁷GpppG or GpppG were
30 added at concentrations of 0.5 mM. A portion of each transcription reaction mixture contained [α -³²P]UTP and the products were analyzed on a 0.4%

polyacrylamide-urea gel to ascertain the presence of a single appropriate size major RNA species. Yields of transcripts were calculated from the absorbance at 260 nm following 2 rounds of spin filtration through
5 Sephadex G-50 columns and 2 ethanol precipitations.
In vitro translation. RNA was translated in a rabbit reticulocyte lysate as described by Promega Biotec except that the final reaction volume was 12.5 μ l; 0.1 mM m^7 GDP was used as a specific inhibitor of cap-
10 dependent translation when indicated. The final concentrations of in vitro or in vivo made RNA in the translation reaction mixture were 8 nM or 80 ng/ μ l, respectively. Following translation, 25 μ l of a solution containing 9 M urea, 75 mM EDTA, 1.5 mM
15 phenylmethyl sulfonyl fluoride, 8000 U/ml RNase T1, and 40 μ l/ml RNase A were added for a further 30 min incubation at 30°C to digest aminoacylated tRNA. A 10 μ l sample of the translation mixture was dissociated with NaDodSO₄ and mercaptoethanol and analyzed by 15%
20 polyacrylamide gel electrophoresis as described by Pelletier et al (1988, Mol. Cell. Biol. 8, 1103-1112).
RNA preparation and analysis. HeLa S-3 cells (5×10^8) were coinfectd with recombinant vaccinia viruses as described by Fuerst et al, supra; 12 hr later, extracts
25 were prepared and sedimented on sucrose gradients as described by Katze et al (1986, J. Virol. 60:1027-1039). Portions of each fraction were used for absorbance measurements at 260nM and for slot blot hybridization (White et al, 1982, J. Biol. Chem 157,
30 8569-8572) with a [³²P]-labeled CAT DNA probe.
Polyribosome- and free RNA-containing fractions were pooled separately, ethanol precipitated and resuspended

in 4 ml of 0.1 M Tris-HCl (pH 7.5)-12 mM EDTA-0.15 M NaCl-0.1% NaDodSO₄. Proteinase K was added to a final concentration of 50 µg/ml and the mixture was incubated for 30 min at 37°C. RNA was extracted 3 times with
5 phenol-chloroform and twice with chloroform, ethanol precipitated, washed with 70% ethanol and resuspended in water. A portion of each sample was analyzed for size by electrophoresis on a 1.4% agarose-formaldehyde gel. The RNA was transferred to a nitrocellulose
10 membrane and hybridized to a [³²P]-labeled CAT DNA probe.

CAT assay. CAT was measured as described by Shaw (1975, Methods in Enzymology 43, 737-748), with modifications. Confluent CV-1 cells in a 6-wells plate
15 were coinfectd and grown with minimum essential medium without Phenol Red (Quality Biological, Inc.) and supplemented with 2.5% fetal bovine serum. At 24 hr after infection, the cells and medium were collected and lysis was achieved by addition of NaDodSO₄ and
20 chloroform to 0.0005% and 1%, respectively. Samples (5-20 µl) were transferred to a 96-wells plate and 0.2 ml of 100 mM Tris-HCl (pH 7.8)/0.1 mM acetyl CoA/0.4 mg/ml 5, 5'-dithiobis-2-nitrobenzoic acid/0.1 mM chloramphenicol was added. After 15 min the absorbance
25 at 412 nm was determined by a microplate reader (Molecular Devices Corp.)

For isolation of the desired protein (CAT in the present example) in a purified form, conventional isolation and purification techniques are employed.
30 Such techniques include centrifugation, filtration, chromatography, electrophoresis, dialysis and the like well known to one of ordinary skill in the art.

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RESULTS

Effect of EMCV UTR on CAT expression. In order to determine whether the EMCV UTR would confer enhanced and cap-independent translation to the predominantly uncapped transcripts made by bacteriophage T7 RNA polymerase, encoded by vaccinia virus, in mammalian cells, two plasmids, pT7CAT and pT7EMCAT, were constructed. Each contained the T7 ϕ 10 promoter and the CAT gene by the latter also had a segment of the EMCV UTR, corresponding to nucleotides 163 to 746 of the EMCV UTR downstream of the T7 RNA start site as shown in Figure 1. CAT activity was measured 24 hr after transfection of each T7 promoter plasmid into cells that had been infected with the helper virus vTF7-3 (Fuerst et al, supra), which express the T7 RNA polymerase. An approximately 7-fold enhancement provided by the EMCV UTR was reproduced in several independent experiments. CAT level expressed by a T7EMCAT plasmid which did not contain the T7 stem-loop was 10 fold less than T7EMCAT indicating that the T7 stem-loop is necessary for expression despite the fact that EMCV UTR is expected to have considerable secondary structure. Both pT7CAT and pT7EMCAT contain vaccinia virus thymidine kinase sequences flanking the expression cassette making it simple to generate recombinant vaccinia viruses vT7CAT and vT7EMCAT. CAT expression was measured after infecting cells simultaneously with either T7 promoter-containing recombinant virus and vTF7-3 (to provide T7 RNA polymerase). The overall results were similar to those obtained by transfection with the corresponding plasmids except that the EMCV UTR enhanced expression

4- to 5-fold in repeated experiments.

Effects of hypertonic medium on expression. In order to determine the effect of hypertonicity on CAT expression, CV-1 cells were infected with vTF7-3 to provide T7 RNA polymerase and either vT7CAT or vT7EMCAT. Protein synthesis was monitored by NaDodSO₄-polyacrylamide gel electrophoresis of cytoplasmic proteins from cells labeled with [³⁵S]methionine at various times after infection in isotonic or hypertonic (190 mM) medium (Figure 2). At 2 hr after infection, predominantly host proteins were labeled under all conditions (lanes 1-4). However, by 6 hr. host protein synthesis was inhibited and the late pattern of protein synthesis was established (lanes 5-8). A band, corresponding in size to the CAT polypeptide, was clearly seen in the autoradiogram of proteins from cells infected with vT7EMCAT (lane 7) but was hardly detected in the proteins from cells infected with vT7CAT (lane 6) and missing from control cells infected with wild-type vaccinia virus (not shown). Moreover, under hypertonic conditions, there was an absolute increase in labeling of CAT and a decrease in labeling of vaccinia viral late proteins, in cells infected with vT7EMCAT (lane 8). At still later times, CAT expressed by vT7EMCAT was readily detected as a prominent band (lane 10) of similar intensity to that of the major viral proteins (lane 9). At this time, however, the CAT protein was the predominant band expressed by vT7EMCAT under isotonic conditions (lane 12) and was even more dominant under hypertonic conditions (lane 13). Densitometer tracings of the fluorogram indicate that at 24 hr. 78% of the [³⁵S]methionine was

incorporated into CAT.

The total accumulation of CAT protein at 24 hr after infection was determined by Coomassie brilliant blue staining of NaDodSO₄-polyacrylamide gels as well as
5 by assaying CAT activity (Figure 3). Cells were co-infected in isotonic medium and then either maintained under these conditions or were transferred to hypertonic medium after 4 hr. After a total of 24 hr, the cells were lysed and 20 µg of cytoplasmic protein
10 were loaded on each lane of a 10% polyacrylamide gel. Following electrophoresis, densitometry of the Coomassie brilliant blue stained gel revealed that the CAT protein comprised 2% or 7% of the total protein when expressed from vT7CAT or vT7EMCAT under isotonic
15 conditions, respectively and 10% of the total protein when expressed from vT7EMCAT in hypertonic conditions (Figure 3A). The selective effect of hypertonicity on EMCV UTR-mediated expression was confirmed by measurements of CAT activity in extracts prepared from
20 cells at 24 hr after co-infection (Figure 3B). CAT activity was 10-fold higher when expressed from vT7EMCAT in hypertonic medium as compared to the expression level from vT7CAT.

Effect of EMCV UTR on in vitro translation properties
25 of T7 transcripts. To further investigate the mechanism by which the EMCV UTR had elevated the expression of the presumably uncapped T7 transcripts in vivo, we synthesized RNAs with and without m⁷G capped 5'-ends and compared their in vitro translation
30 properties. Bacteriophage T7 RNA polymerase was used to transcribe the plasmids pT7CAT and pT7EMCAT (Figure 1) in order to prepare mRNAs with and without the EMCV

UTR. Transcripts containing 5' methylated caps were generated by adding high concentrations of m⁷GpppG to the transcription reaction; GpppG was added to parallel reactions to generate unmethylated caps which are known to be non-functional in translation but may also protect the RNAs against exonuclease degradation. These transcripts were translated in a rabbit reticulocyte lysate in the presence or absence of the cap analog m⁷GDP, known to be a specific inhibitor of methylated capped mRNA translation in vitro. In the absence of the EMCV UTR, translation of T7-CAT transcripts was found to be methylated cap-dependent as the GpppG-terminated RNA was inefficiently translated relative to that of m⁷GpppG-terminated RNA (Figure 4). Moreover, translation of m⁷GpppG-terminated RNA was inhibited by m⁷GDP (Figure 4). In contrast, the translation of T7-EMCV UTR-CAT transcripts was highly efficient regardless of 5' cap structure and was insensitive to m⁷GDP as well, indicating that the EMCV UTR-containing mRNAs were translated in a cap-independent fashion. The specificity of m⁷GDP was further demonstrated by simultaneously translating capped transcripts, with and without EMCV UTRs, that encode different length polypeptides; only those without the EMCV UTR were inhibited (data not shown).

Association of T7 transcripts with polyribosomes. If the beneficial effect of the EMCV UTR were due to more efficient translation of uncapped messages, as the in vitro studies had suggested, than a higher percentage of such transcripts should be polyribosome-associated relative to that of T7 transcripts lacking the UTR. To evaluate this, Hela cells were co-infected with vTF7-3

and either vT7CAT or vT7EMCAT and 12 hr later cytoplasmic extracts were prepared and subjected to sucrose density gradient centrifugation. The monosomes peak was located by optical density measurements and the distribution of CAT mRNA along the gradient was determined by hybridization of a sample of each fraction with a [^{32}P]-CAT probe. Autoradiograms were made of the "slot-blot" hybridizations and the intensity of each slot was measured by densitometry. These data, compiled in Figure 5A, revealed that there were similar total amounts of T7-EMCV UTR-CAT RNA and T7-CAT RNA but that a larger fraction of the former was associated with disomes and heavier polyribosomes.

The polyribosome-associated and the free CAT RNA were pooled separately and translated in rabbit reticulocyte lysates in the presence or absence of m⁷GDP. Inspection of fluorograms of NaDodSO₄-polyacrylamide gels (Figure 5B) led to several significant findings. First, both the polyribosome-bound and free T7-EMCV UTR-CAT RNA was translated more efficiently than either T7-CAT RNA fraction. Second, translation of the polyribosome-bound T7-CAT RNA was inhibited by m⁷GDP whereas the corresponding free RNA was not inhibited. Third, translation of neither the polyribosome nor the free T7 EMCV UTR-CAT RNA was inhibited by m⁷GDP. Without being bound to any specific theory, these observations were interpreted as follows. The small amounts of capped T7-CAT transcripts were selectively associated with the polyribosome fraction accounting for the inhibition of their translation by m⁷GDP. The free T7-CAT RNA was mostly uncapped but owing to the large amount it was translated, albeit

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inefficiently, and in a cap-independent manner. The cap-status of the T7 EMCV UTR-CAT RNA could not be judged since our in vitro studies had shown that neither capped nor uncapped forms are inhibited by m⁷GDP. However, since the 5' ends of the T7-CAT and T7-EMCV UTR-CAT transcripts contain the identical 5' end and stem-loop structure, there is no reason to assume that they would be differentially capped.

Studies have indicated that the effective utilization of prokaryotic transcription components in a eukaryotic cell raises a number of potential problems, the solution of which may provide not only a superior expression system, but also could contribute to the understanding of eukaryotic RNA structure/function relationships. The bacteriophage T7 transcription system, or similar ones from related bacteriophages, offer advantages that have been previously enumerated (Fuerst et al, supra). By choosing a vaccinia virus vector, which replicates in the cytoplasm, extra difficulties associated with nuclear processing and transport were avoided. Although vaccinia virus has its own capping and methylating enzymes (Wei et al, 1974, Proc. Natl. Acad. Sci. USA 71, 3014-3018), RNA made by T7 RNA polymerase is not as efficiently capped as RNAs made by the vaccinia virus polymerase (Fuerst et al, supra). Since the cap is needed for both RNA stability and translation [Grifo et al, 1982, in Interaction of Translational & Transcriptional Controls in the Regulation of Gene Expression eds. Grunberg-Manago, M. & Safer, B., (New York: Elsevier Biomedical), pp. 359-372], this deficiency presented a severe challenge.

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The RNA stability problem, however, was solved by retaining the T7 stem-loop segment at the 5' ends of the transcripts. This stem-loop, nevertheless, may have exacerbated the capping problem since in vitro studies indicated that RNA containing this structure was a poorer substrate for the vaccinia RNA guanylyl transferase (Fuerst et al, supra). In addition, the stem-loop structure might interfere with ribosome scanning (Pelletier et al, 1985, Cell 40, 515-526; Kozak, M., 1980, Cell 19, 79-90). Nevertheless, owing to the enormous amount of stable RNA generated (approximately 30% of the total cytoplasmic RNA), useful amounts of protein were made with the vaccinia/T7 hybrid expression system.

It is important to point out here that at the start of the present study, the applicants did not know whether the EMCV UTR could function in a eukaryotic cell when attached to an RNA made by a prokaryotic transcription system. Further more, since vaccinia virus inhibits host cell protein synthesis, it was a matter of concern that the changes in the protein synthesis machinery of the cell caused by vaccinia virus might prevent the efficient use of the EMCV UTR. Hence, when it was found that not only did the EMCV UTR system of the present invention function in the eukaryotic system, but it also enhanced the level of protein made by 500% or more, it was indeed quite unexpected and represented a major improvement in gene expression technology.

The mammalian cell expression system of the present invention is truly eclectic in that the vector is a DNA virus, the RNA polymerase and promoter are

derived from a DNA bacteriophage, and the ribosome binding site is from an RNA virus. Nevertheless, the system provides a powerful, rapid, versatile and simple way of expressing genes in mammalian or avian cells.

- 5 It may be used most simply by transfecting a plasmid containing a T7 promoter regulated gene of interest into cells that have been infected with a standard recombinant vaccinia virus expressing T7 RNA polymerase. In the latter approach, there is no need
- 10 to prepare a new recombinant vaccinia virus. Alternatively, by recombining the T7 promoter regulated gene into vaccinia virus and using a standard double infection protocol, larger numbers of cells may be conveniently used and still higher expression may be
- 15 obtained. Results with the small 25,000 dalton CAT protein, usually chosen for model studies, revealed that it was the dominant polypeptide made in vaccinia virus-infected cells and after only 24 hr accumulated to a level of 16.5 μg per 2×10^6 cells which accounted
- 20 for approximately 10% of the Coomassie brilliant blue stained protein on polyacrylamide gels. The level of expression with the vaccinia/T7/EMCV hybrid system is greater than that obtained with conventional vaccinia virus vectors even when the strongest poxvirus
- 25 promoters described thus far are used (Falkner, et al, 1988, J. Virol. 62, 1849-1854; Patel et al, 1988, Proc. Natl. Acad. Sci. USA 85, 9431-9435). The vaccinia hybrid system has other advantages, such as use for potentially toxic genes, since expression does not
- 30 occur until cells are co-infected with the T7 RNA polymerase containing helper virus. Thus, the expression of toxic genes to kill target cells can be

controlled by this unique feature of the present invention.

Of course, having demonstrated the operability of the present invention with the model CAT system, it
5 is clear that the expression of other proteins is achieved similarly, as will be suggested to one of ordinary skill in the art.

It is noted that the embodiments described herein are for illustrative purposes only and that
10 various modifications in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

WHAT IS CLAIMED IS:

1. A recombinant vaccinia virus, the genome of which includes a T7 promoter followed by a cDNA copy of an untranslated region of a cardiovirus RNA that confers cap independent translation, followed by a gene encoding a protein desired to be expressed, said promoter, cDNA and the gene being so arranged that said gene is efficiently expressed in a mammalian cell infected with said recombinant virus only when said cell contains T7 RNA polymerase.
2. The recombinant virus of claim 1 wherein said untranslated region is from EMCV.
3. A recombinant plasmid comprising a T7 promoter followed by a cDNA copy of an untranslated region of a cardiovirus RNA that confers cap independent translation, followed by a gene encoding a protein desired to be expressed, said promoter, cDNA and the gene being so arranged that said gene is efficiently expressed in a mammalian cell infected with said recombinant virus only when said cell contains T7 RNA polymerase.
4. The plasmid of claim 3 wherein said untranslated region is from EMCV.
5. The plasmid of claim 4 having ATCC accession number 68045.
6. A method for synthesizing protein, comprising infecting a cell with the vaccinia virus of claim 1 or claim 2 and infecting said cell with a second vaccinia virus that expresses T7 RNA polymerase and then recovering the desired protein by suitable isolation and purification techniques.
7. A method for synthesizing protein, which comprises transfecting a cell with the plasmid of claim 3 or claim 4 and infecting said cell with a DNA based cytoplasmic virus expressing T7 RNA polymerase, and then recovering the desired protein by suitable isolation and purification techniques.
8. The method of claim 6 further exposing said cell to hypertonic conditions.
9. The method of claim 7 further exposing said cell to hypertonic conditions.
10. The method of claim 6 wherein said gene is a toxic gene.
11. The method of claim 7 wherein said gene is a toxic gene.
12. The method according to claim 8 of infecting with said DNA based cytoplasmic virus at a chosen time.



13. The method according to claim 9 of infecting said DNA based cytoplasmic virus at a chosen time.
14. The recombinant virus of claim 1 or claim 2 expressing the desired protein in an amount about 500% more than the amount expressed without said untranslated region being present in the genome of said recombinant vaccinia virus.
15. A method for obtaining an enhanced amount of a desired protein, comprising employing the plasmid of claim 3 or claim 4 to construct a recombinant vector and then isolating the protein expressed by said vector in a suitable cellular medium by conventional isolation and purification techniques, the amount of protein thus obtained being about 500% more than the amount obtained without employing the untranslated region.
16. A recombinant vaccinia virus substantially as herein described with reference to the example.
17. A recombinant vaccinia virus substantially as herein described with reference to Fig.1.
18. A method for synthesizing protein, comprising infecting a cell with the vaccinia virus of claim 16 or claim 17 and infecting said cell with a second vaccinia virus that expresses T7 RNA polymerase and then recovering the desired protein by suitable isolation and purification techniques.

Dated 19 April, 1993

The United States of America

Patent Attorneys for the Applicant/Nominated Person

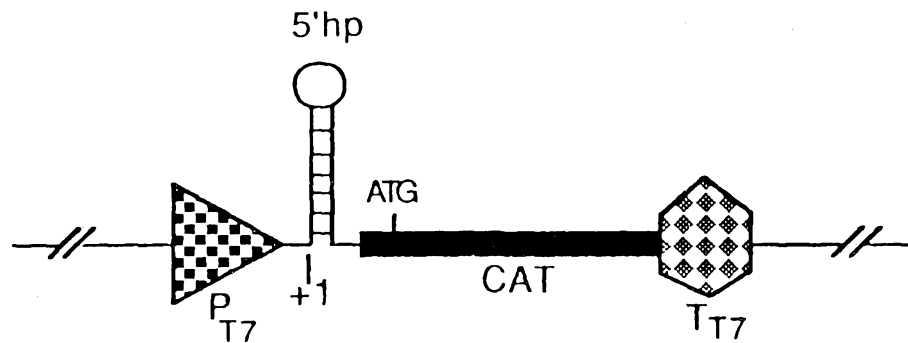
SPRUSON & FERGUSON



IG:WPUERWJBTJ00267:EAR

FIG. 1

pT7CAT



pT7EMCAT

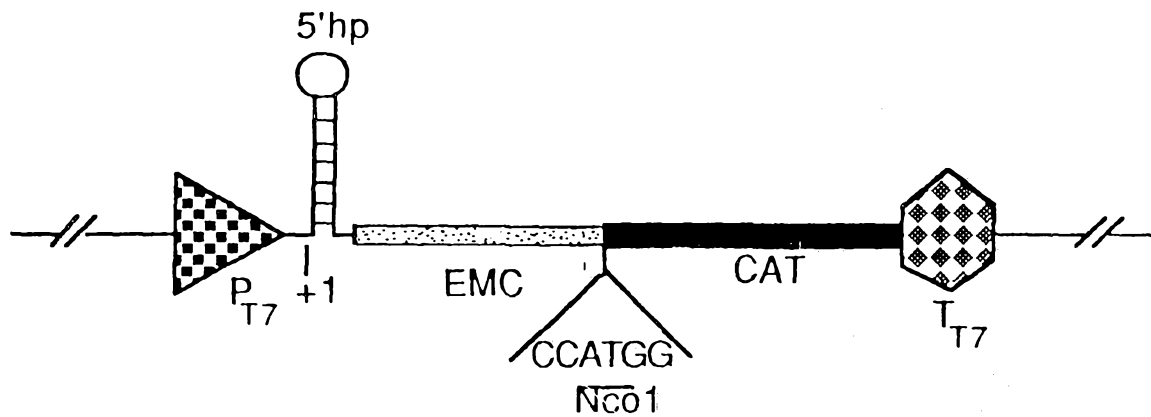
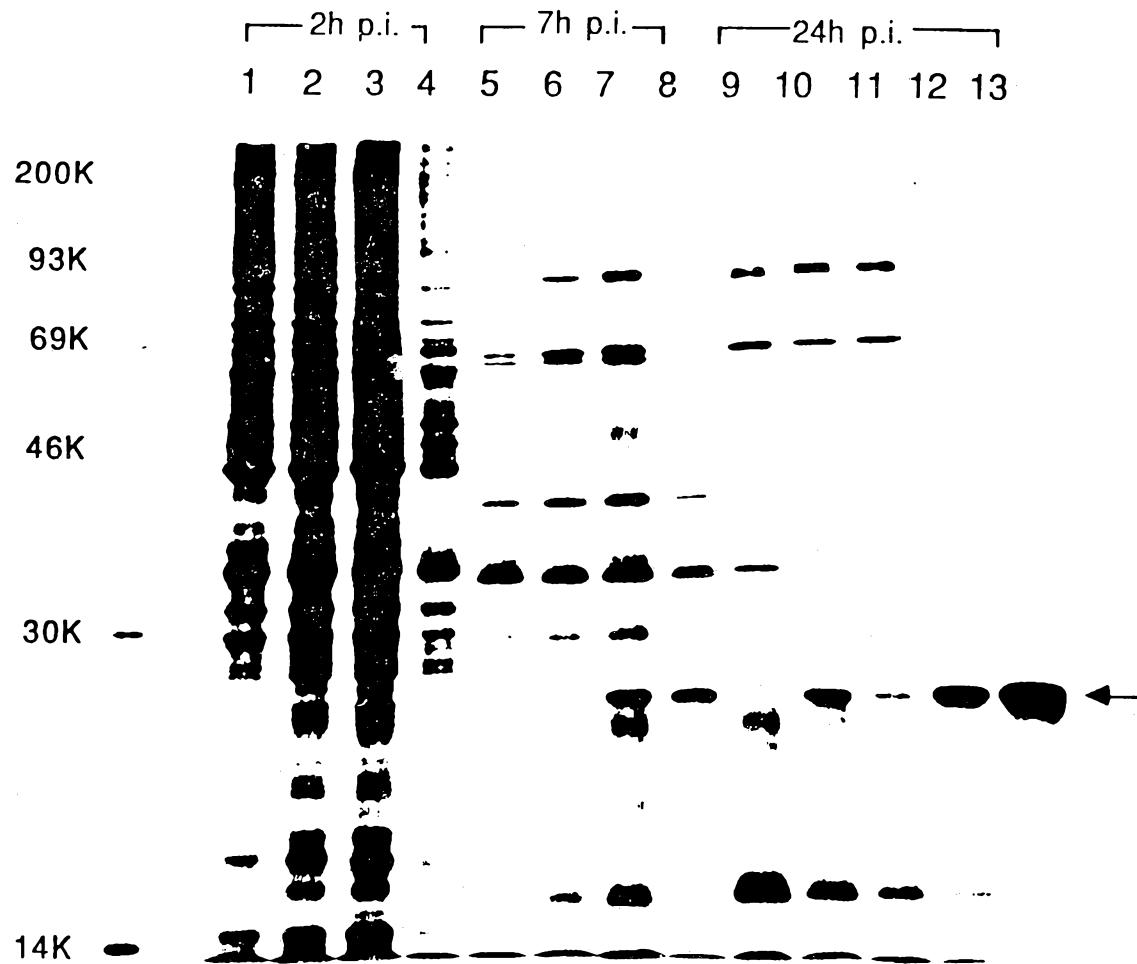


FIG. 2



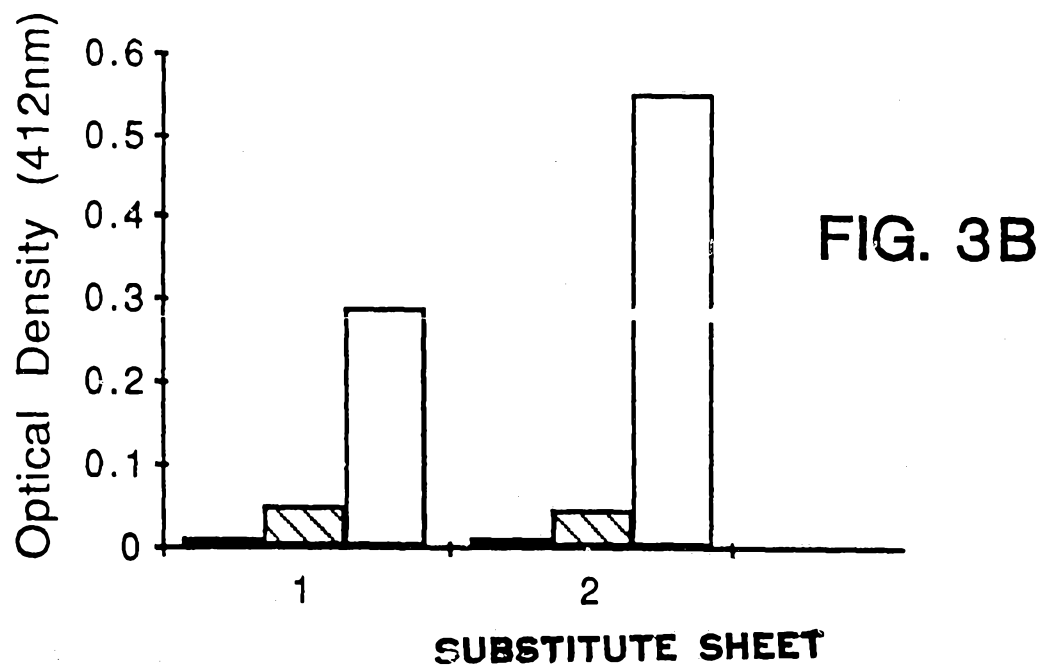
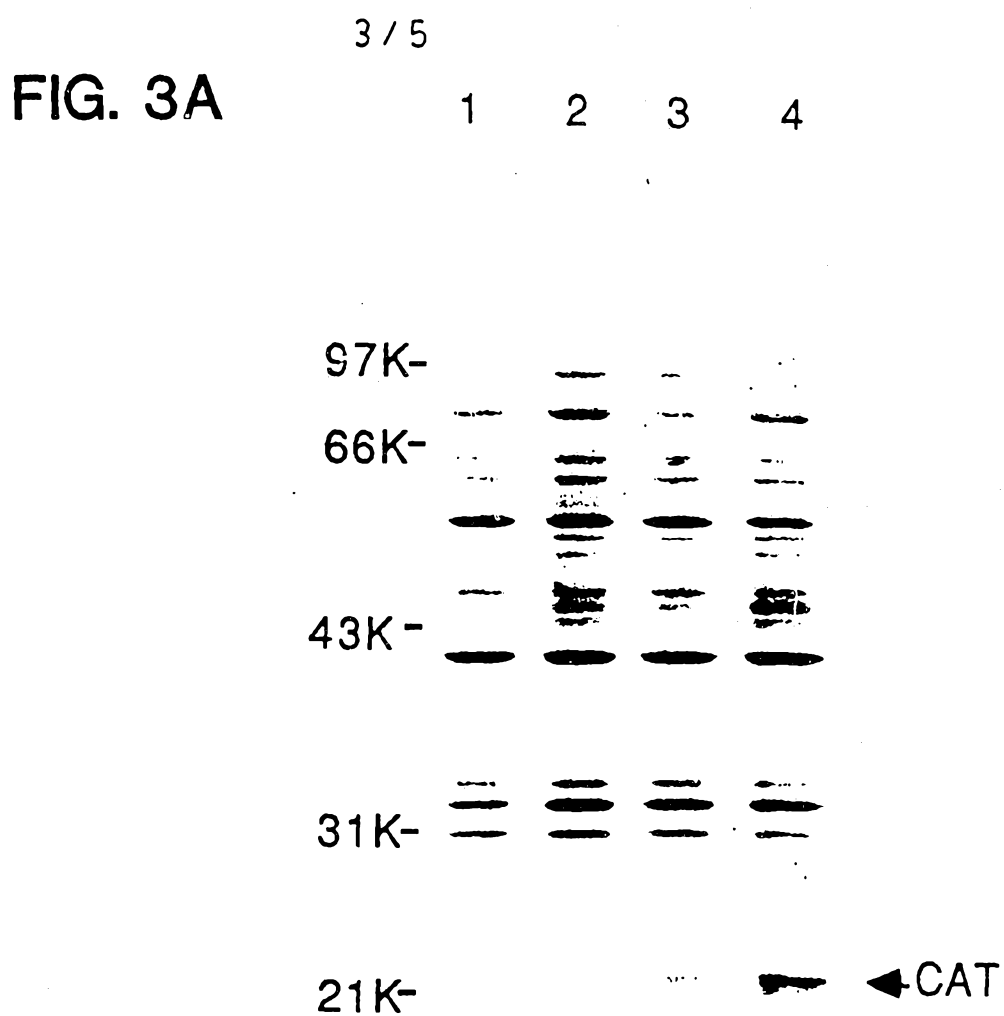


FIG.4 1 2 3 4 5 6 7 8

200K

93K

69K

46K

30K



14K

GpppG	+	+			+	+		
m ⁷ GpppG			+	+			+	+
m ⁷ GDP		+		+		+		+

5 / 5

FIG. 5A

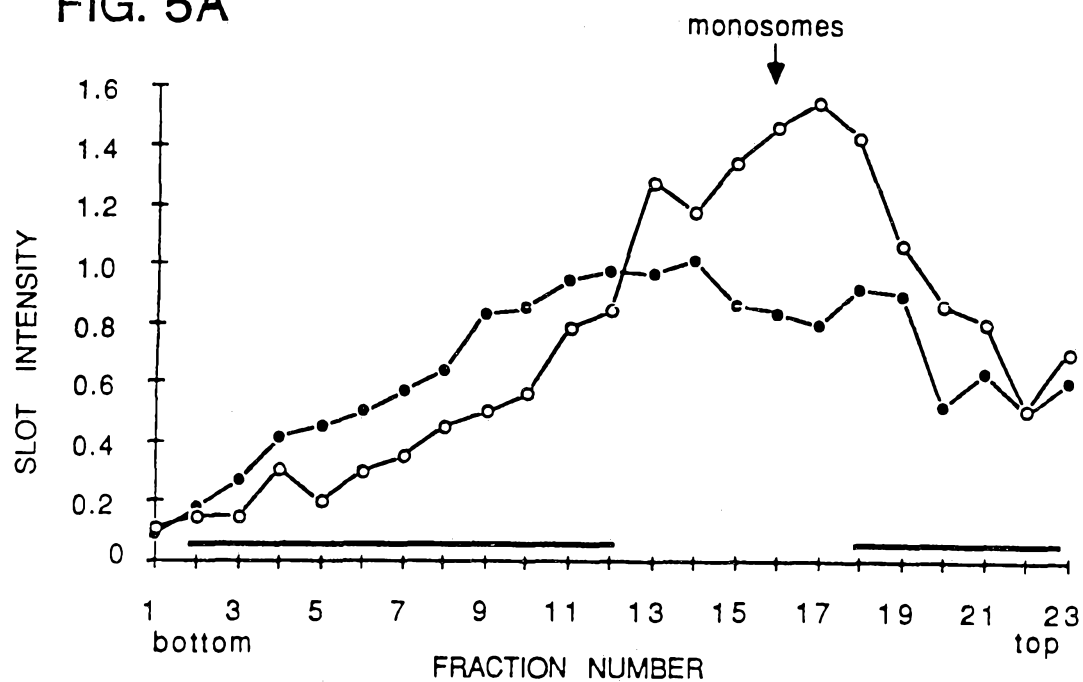
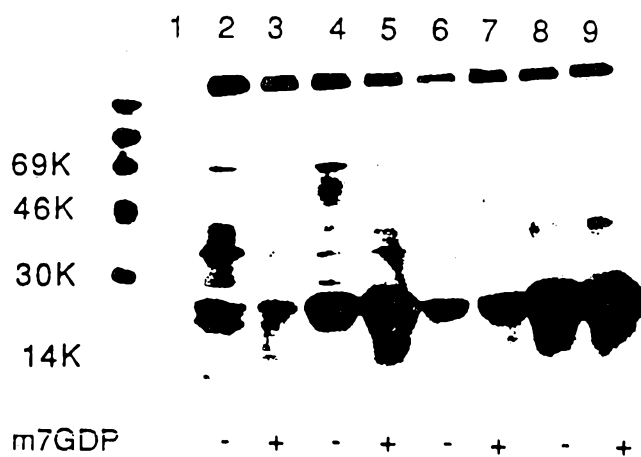


FIG. 5B



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INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/03687

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ¹		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ⁵ C12N 7/01, 15/00, 15/67, 15/86		
US 435/69.1, 320, 235, 172.3		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Class. No. of Document	
US	435/69.1, 320, 235, 172.3 935/27, 32, 34, 60	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵		
DATABASES: CAS AND APS		
KEY WORDS: PHAGE RNA POLYMERASES, EUCARYOTIC CELLS, TRANSLATIONAL ENHANCERS		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category [*]	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	Journal of Virology, Vol. 60, No. 2, issued November 1986, Parks et al., Encephalomyocarditis Virus 3C Protease: Efficient Cell-Free Expression from clones which Link Viral 5' Noncoding Sequences to the P3 Region", pages 376-384, see entire document.	1-15
X	Proceedings of the National Academy of Sciences, Vol. 83, issued November 1986, Fuerst et al., "Eucaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase", pages 8122-8126, see entire document.	1-15
[*] Special categories of cited documents: ¹⁵ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ²	
27 September 1990	30 NOV 1990	
International Searching Authority ¹	Signature of Authorized Officer ²⁰	
ISA/US	Sharon Nolan	