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Cap-independent translation of mRNA conferred by encephalomyocarditis virus 5′ sequence improves the performance of the vaccinia virus/bacteriophage T7 hybrid expression system

(mammalian expression vector/genetic engineering/poxvirus)

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ABSTRACT **A recombinant vaccinia virus that directs the synthesis of bacteriophage T7 RNA polymerase provides the basis for the expression of genes that are regulated by T7 promoters in mammalian cells. The T7 transcripts, which account for as much as 30% of the total cytoplasmic RNA at 24 hr after infection, are largely uncapped. To improve the translatability of the uncapped RNA, the encephalomyocarditis virus (EMCV) untranslated region (UTR) was inserted between the T7 promoter and the chloramphenicol acetyltransferase (CAT) gene. Experiments with a reticulocyte extract demonstrated that the EMCV UTR conferred efficient and cap-independent translatability to CAT RNA synthesized *in vitro* by T7 RNA polymerase. In cells infected with recombinant vaccinia viruses containing the T7 promoter-regulated CAT gene, the EMCV UTR increased the amount of CAT RNA on polyribosomes. The polyribosome-derived CAT RNA, which contained the EMCV UTR, was translated *in vitro* in a cap-independent fashion as well. Use of the EMCV UTR significantly enhanced the vaccinia/T7 hybrid expression system as it resulted in a 4- to 7-fold increase in total CAT activity. A further ≈2-fold improvement was achieved by incubating the cells in hypertonic medium, which favors the translation of uncapped picornavirus RNA over cellular mRNAs. With this newly modified expression system, CAT was the predominant protein synthesized by infected cells and within 24 hr accounted for >10% of the total cell protein.**

A eukaryotic expression system based on a recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase in the cytoplasm of infected mammalian cells was described previously (1–3). The target gene for the bacteriophage RNA polymerase, flanked by T7 promoter and termination sequences, was introduced into infected cells either by transfection of a recombinant plasmid or by infection with a second recombinant vaccinia virus. Through the use of transfection or infection protocols, it was found that T7 lacZ transcripts comprise 10% or 30%, respectively, of the total cytoplasmic RNA at 24 hr after infection. The T7 transcripts were initiated correctly, but only 5–10% contained 5′-terminal cap structures, providing an explanation for the discrepancy between the major amount of RNA made and the moderate amount of protein expressed. The more efficient capping of vaccinia virus mRNA, compared to T7 RNA, may be due to association of the viral RNA guanylyltransferase with the viral RNA polymerase (4). In addition, the 5′ end stem–loop structure of the T7 transcripts, which was found to be crucial for its stability (3), might interfere with capping as well as with ribosome binding and scanning.

To improve the translatability of the uncapped T7 transcripts, we considered two alternatives: increase the percentage of RNA capped or confer cap-independent translatability to the RNA. With regard to the latter, RNAs of several viruses including members of the picornavirus family are naturally uncapped yet are translatable in eukaryotic cells. Of these, encephalomyocarditis virus (EMCV) RNA is known to be a particularly active messenger (5, 6). In addition to lacking a cap, picornavirus RNAs have a long 5′ untranslated region (UTR) (650–1300 nucleotides) with numerous AUG codons preceding the initiator codon for the polyprotein (7). It has been suggested that poliovirus and EMCV RNAs contain a ribosomal binding site within the UTR that allows cap-independent translation (8–11). In contrast, the ribosome-scanning model of translational initiation has been accepted for the vast majority of eukaryotic mRNAs (12, 13). An essential feature of this model is that the 40S ribosomal subunit binds only to the 5′ end cap structure and usually initiates at the first AUG.

In this communication, we describe the use of the EMCV UTR and hypertonic conditions for cap-independent translation of T7 transcripts expressed by recombinant vaccinia viruses in mammalian cells. These modifications provide higher levels of translation products than previously obtained with the vaccinia/T7 hybrid expression system or with more conventional vaccinia virus vectors.

MATERIALS AND METHODS

Plasmid and Virus Constructions. A 583-base-pair (bp) *Eco*RI/*Bal* I EMCV fragment from pE5LVPO (14) containing nucleotides 163–746 of the EMCV genome was inserted into the blunt-ended *Bam*HI site of plasmid vector pAR2529 (15) to generate pTF7.25EMC-1. The chloramphenicol acetyltransferase (CAT) coding sequence was isolated from pTF7CAT-1 (1), as a 0.8-kilobase-pair (kbp) *Taq* I fragment and through the use of *Bam*HI linkers was inserted into the *Bam*HI site of pUC18 to generate the plasmid pUC18CAT-1. The 0.8-kbp *Bam*HI CAT fragment was then isolated from pUC18CAT-1 and inserted into a unique *Bam*HI site of plasmid pTF7.25EMC-1 downstream of the major translation start site of EMCV, to form plasmid pTF7.25EMCAT-1. This resulted in placement of the CAT translation start site in-frame with the EMCV major start signal separated by 12 codons. The coding frame of CAT was placed immediately downstream of the EMCV start codon by site-directed mutagenesis. The sequence immediately flanking the initiating EMCV ATG was changed to 5′-EMCV. . . GATAATAC-CATGGAG. . .CAT-3′, which provides a unique *Nco* I site. An *Nco* I site contained within the CAT gene was removed

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Abbreviations: EMCV, encephalomyocarditis virus; UTR, untranslated region; CAT, chloramphenicol acetyltransferase.

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by placing a silent mutation within the coding sequence. The mutagenized EMCV CAT cassette was then isolated as a 0.82-kbp *Bam*HI/*Kpn* I fragment and inserted into the corresponding site in plasmid pTF7.25EMCAT-1 to create pTF7.25EMCAT-20. For simplicity, pTF7.25EMCAT-20 and pTF7CAT-1 are referred to as pT7EMCAT and pT7CAT, respectively. Vaccinia virus (strain WR) insertion vectors were prepared by excising the complete T7 promoter-target gene-T7 terminator cassettes as *Bgl* II fragments, blunting the ends, and inserting them into the blunt-ended *Eco*RI site of pGS50 (2). This resulted in flanking the target gene cassettes with vaccinia virus thymidine kinase sequences used for homologous recombination (16).

***In Vitro* Transcription.** Plasmids were digested with *Bam*HI to release the T7 transcription unit as a linear DNA fragment. Transcription was carried out with T7 RNA polymerase as recommended by Promega Biotec except that the GTP concentration was 0.05 mM and m^7GpppG or GpppG (Pharmacia) was added at 0.5 mM. A portion of each transcription reaction mixture contained [α-^{32}P]UTP and the products were analyzed on a 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 after two rounds of spin filtration through Sephadex G-50 columns and two 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 μl; 0.1 mM m^7GDP was used as a specific inhibitor of cap-dependent translation when indicated. The final concentrations of *in vitro* or *in vivo* made RNA in the translation reaction mixture were 8 nM or 40 ng/μl, respectively. After translation, 25 μl of a solution containing 9 M urea, 75 mM EDTA, 1.5 mM phenylmethylsulfonyl fluoride, 8000 units of RNase T1 per ml, and 40 μg of RNase A per ml was added for a further 30-min incubation at 30°C to digest aminoacylated tRNA. A 10-μl sample of the translation mixture was dissociated with $NaDodSO_4$ and mercaptoethanol and analyzed by 15% polyacrylamide gel electrophoresis (11).

RNA Preparation and Analysis. HeLa S-3 cells (5×10^8) were coinfected with recombinant vaccinia viruses as described (3); 12 hr later, extracts were prepared and sedimented on sucrose gradients (17). Portions of each fraction were used for absorbance measurements at 260 nm and for slot blot hybridization (18) with a ^{32}P-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% $NaDodSO_4$. 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 three times with phenol/chloroform and twice with chloroform, ethanol precipitated, washed with 70% ethanol, and resuspended in water. A portion of each sample was analyzed by electrophoresis on a 1.4% agarose/formaldehyde gel. The RNA was transferred to a nitrocellulose membrane and hybridized to a ^{32}P-labeled CAT DNA probe.

CAT Assay. CAT was measured as described (19), with the following modifications: confluent CV-1 cells in a 6-well plate were coinfected and grown with minimum essential medium without phenol red (Quality Biologicals, Gaithersburg, MD) 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 $NaDodSO_4$ and chloroform to 0.0005% and 1%, respectively. Samples (5–20 μl) were transferred to a 96-well plate and 0.2 ml of 100 mM Tris·HCl, pH 7.8/0.1 mM acetyl-CoA/5,5′-dithiobis-2-nitrobenzoic acid (0.4 mg/ml)/0.1 mM chloramphenicol was added. After 15 min, the absorbance at 412 nm was determined by a microplate reader (Molecular Devices, Menlo Park, CA).

RESULTS

Effect of EMCV UTR on CAT Expression. Our objective was to determine whether the EMCV UTR would confer enhanced and cap-independent translation to the predominantly uncapped transcripts made by vaccinia virus-encoded bacteriophage T7 RNA polymerase in mammalian cells. Two plasmids, pT7CAT and pT7EMCAT, were constructed. Each contained the T7 ϕ10 promoter and the CAT gene but the latter also had a segment of the EMCV UTR, as shown in Fig. 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 (1), which expresses the T7 RNA polymerase. An ≈7-fold enhancement provided by the EMCV UTR was reproduced in several independent experiments. The CAT activity expressed by a plasmid with the EMCV UTR but without the T7 stem–loop was 10-fold less than T7EMCAT (data not shown), indicating that the T7 stem–loop is still necessary despite the fact that EMCV UTR is expected to have considerable secondary structure.

Recombinant vaccinia viruses vT7CAT and vT7EMCAT were produced from pT7CAT and pT7EMCAT, respectively, using procedures similar to those previously described (16).

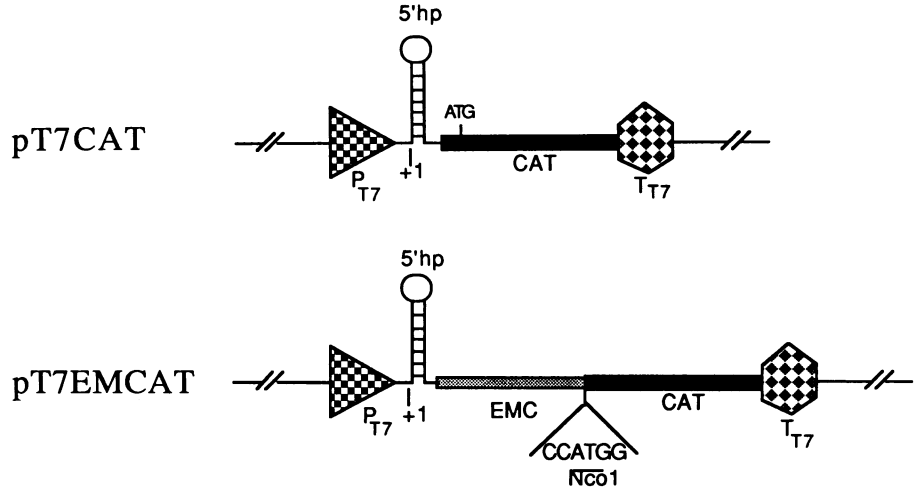

FIG. 1. Structure of expression cassettes. P_{T7}, bacteriophage T7 ϕ10 promoter from −23 to +1; 5′hp, nucleotides +1 to +26 downstream of the ϕ10 promoter and capable of forming a hairpin or stem–loop structure; T_{T7}, bacteriophage T7 ϕ10 termination sequence; EMC, nucleotides 163–746 of the modified EMCV UTR.

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. Hypertonic conditions have been reported to selectively enhance cap-independent translation of picornavirus RNA (20). We reasoned that if the EMCV UTR was improving CAT expression by means of cap-independent translation of the T7 transcripts, then hypertonic medium might provide a further enhancement. Accordingly, CV-1 cells were infected with vTF7-3 to provide T7 RNA polymerase and either vT7CAT or vT7EMCAT. Protein synthesis was monitored by $NaDodSO_4$/polyacrylamide gel electrophoresis of cytoplasmic proteins from cells labeled with [^{35}S]methionine at various times after infection in isotonic or hypertonic (190 mM) medium (Fig. 2). At 2 hr after infection, predominantly host proteins were labeled under all conditions (lanes 1–4). However, by 7 hr, host protein synthesis was inhibited and the late pattern of viral protein synthesis was established (lanes 5–8). A band, corresponding in size to the CAT polypeptide, was clearly seen among the proteins from cells infected with vT7EMCAT (lane 7) but was hardly detected in the proteins from cells infected with vT7CAT (lane 5) and was absent from control cells infected with wild-type vaccinia virus (data 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), whereas there was no enhancement with vT7CAT (lane 6). At still later times, CAT expressed by vT7CAT was readily detected as a prominent band under isotonic conditions (lane 10) but was diminished in intensity under hypertonic conditions (lane 11). At the same 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 lane 13 of the fluorogram indicated that at 24 hr, 78% of the [^{35}S]methionine was incorporated into CAT.

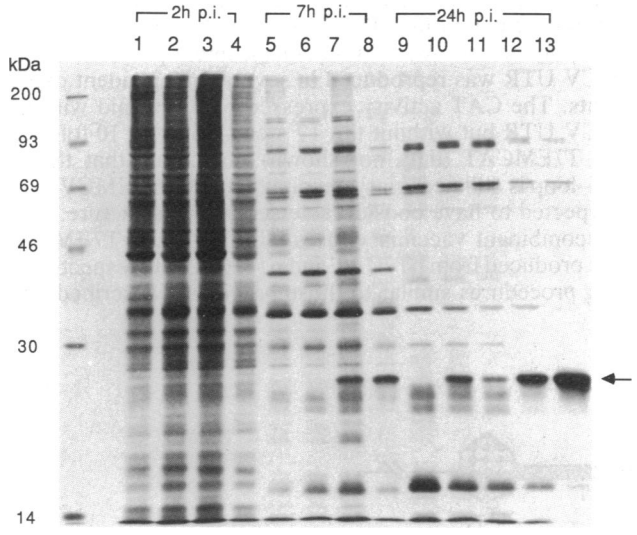

FIG. 2. Time course of CAT synthesis during vaccinia virus infection. CV-1 cell monolayers were infected with 20 plaque-forming units (pfu) per cell of wild-type vaccinia virus (lane 9), coinfected with vTF7-3 and vT7CAT (lanes 1, 2, 5, 6, 10, and 11), or coinfected with vTF7-3 and vT7EMCAT (lanes 3, 4, 7, 8, 12, and 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 [^{35}S]methionine for 30 min. Starvation and pulse labeling were done in isotonic (lanes 1, 3, 5, 7, 9, 10, and 12) or hypertonic (190 mM NaCl) (lanes 2, 4, 6, 8, 11, and 13) conditions. Cell lysates were prepared and equal volumes (corresponding to 2×10^5 cells) were loaded in each lane of a 10% $NaDodSO_4$/polyacrylamide gel. After electrophoresis, the gel was processed for fluorography. The arrow identifies the CAT protein. p.i., Postinfection.

The total accumulation of CAT protein at 24 hr after infection was determined by Coomassie brilliant blue staining of $NaDodSO_4$/polyacrylamide gels as well as by assaying CAT activity (Fig. 3). Cells were coinfected in isotonic medium and were 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 was loaded on each lane of a 10% polyacrylamide gel. After electrophoresis, densitometry of the stained gel revealed that the CAT protein comprised 2% or 7% of the total protein when expressed from vT7CAT or vT7EMCAT under isotonic conditions, respectively, and 10% of the total protein when expressed from vT7EMCAT in hypertonic conditions (Fig. 3*A*). The selective effect of hypertonicity on EMCV UTR-mediated expression was confirmed by measurements of CAT activity in extracts prepared from cells at 24 hr after coinfection (Fig. 3*B*). CAT activity was 10-fold higher when expressed from vT7EMCAT in hypertonic medium as compared to the expression from vT7CAT.

Effect of EMCV UTR on *in Vitro* Translation Properties 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 m^7G capped 5′ ends and compared their *in vitro* translation properties. Bacteriophage T7 RNA polymerase was used to transcribe the plasmids pT7CAT and pT7EMCAT (Fig. 1) to prepare mRNAs with and without the EMCV UTR. Transcripts containing 5′ methylated caps were generated by adding high concentrations of m^7GpppG to the transcription reaction; GpppG was added to parallel reactions to generate unmethylated caps, which are known to be nonfunctional in translation but may also protect the RNAs

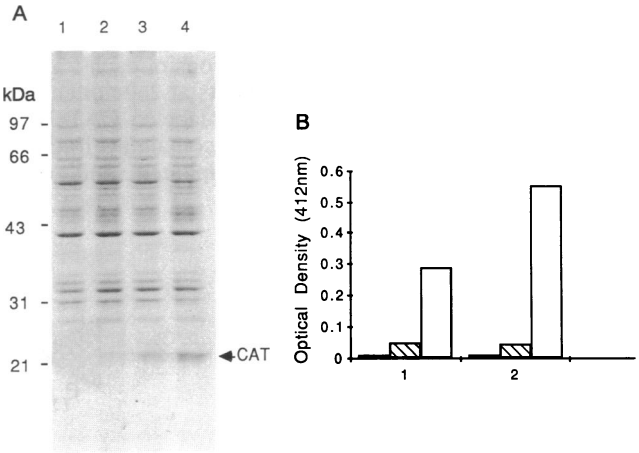

FIG. 3. Accumulation of CAT protein by 24 hr after infection. CV-1 cell monolayers were infected with 20 plaque-forming units (pfu) per cell of wild-type vaccinia virus (*A*, lane 1; *B*, solid bar), coinfected with vTF7-3 and vT7CAT (*A*, lane 2; *B*, hatched bar), or coinfected with vTF7-3 and vT7EMCAT (*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. (*A*) Twenty micrograms of protein was loaded in each lane of a 10% $NaDodSO_4$/polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie brilliant blue. (*B*) CAT activity also was determined on the cell lysates.

against exonuclease degradation. These transcripts were translated in a rabbit reticulocyte lysate in the presence or absence of the cap analog m^7GDP, known to be a specific inhibitor of methylated capped mRNA translation *in vitro* (21). 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 m^7GpppG-terminated RNA (Fig. 4). Moreover, translation of m^7GpppG-terminated RNA was inhibited by m^7GDP (Fig. 4). By contrast, the translation of T7 EMCV UTR CAT transcripts was highly efficient regardless of 5′ cap structure and was insensitive to m^7GDP as well, indicating that the EMCV UTR-containing mRNAs were translated in a cap-independent fashion. The specificity of m^7GDP 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, then 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 coinfected with vTF7-3 and either vT7CAT or vT7EMCAT, and 12 hr later cytoplasmic extracts were prepared and subjected to sucrose density gradient centrifugation. The monosome 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-labeled CAT probe. Autoradiograms were made of the slot-blot hybridizations and the intensity of each slot was measured by densitometry. These data, compiled in Fig. 5*A*, 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 m^7GDP. Inspection of fluorograms of polyacrylamide gels (Fig. 5*B*) led to several significant findings. First, CAT was the major product of the total polyribosome-bound RNA as well as free RNA isolated from vT7EMCAT-infected cells, consistent with the metabolic labeling *in vivo*. Second, both fractions of vT7 EMCV UTR RNA were translated more efficiently than either T7 CAT RNA fraction. Third, translation of the polyribosome-bound T7 CAT RNA was inhibited by m^7GDP, whereas the corresponding free RNA was not inhibited. Fourth, translation of neither the polyribosome nor the free T7 EMCV UTR CAT

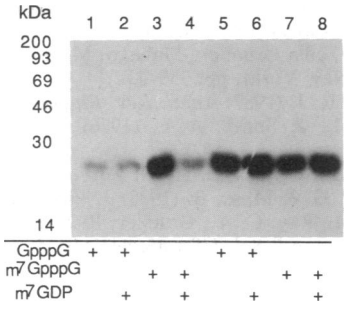

FIG. 4. Cap-independent translation of RNA containing EMCV UTR. *In vitro* transcription was carried out with *Bam*HI-digested pT7CAT (lanes 1–4) or pT7EMCAT (lanes 5–8) as templates. Lanes containing unmethylated (GpppG) or methylated (m^7GpppG) capped RNA are indicated. Translation in micrococcal nuclease-treated rabbit reticulocyte lysate was performed with an RNA concentration of 8 nM with or without the presence of 0.1 mM m^7GDP as indicated.

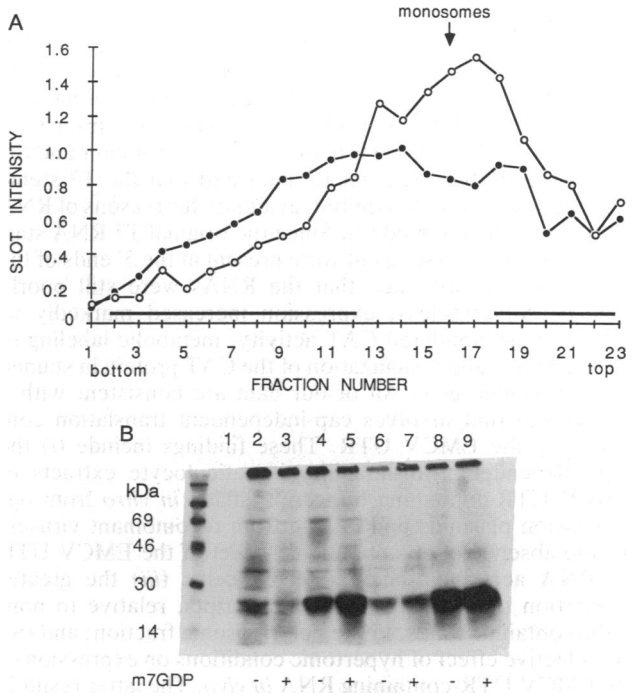

FIG. 5. Polyribosome-association and *in vitro* translation of CAT RNA made in infected cells. (*A*) HeLa cells were coinfected with 10 plaque-forming units (pfu) of vTF7-3 per cell and 10 pfu of either vT7CAT (○) or vT7EMCAT (●) per cell. After 12 hr, cytoplasmic extracts were prepared and sedimented 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-μl sample of each fraction was immobilized on nitrocellulose using a slot blot apparatus and then hybridized to a ^{32}P-labeled CAT probe. Autoradiograms were made and the intensities of the bands were determined by densitometry. Indicated fractions of a similar experiment (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^7GDP as indicated. The translation products of no RNA (lane 1), polyribosome-associated T7 CAT RNA (lanes 2 and 3), polyribosome-associated T7 EMCV UTR CAT RNA (lanes 4 and 5), free T7 CAT RNA (lanes 6 and 7), and free T7 EMCV UTR CAT RNA (lanes 8 and 9) were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and a fluorogram was made.

RNA was inhibited by m^7GDP. We interpreted these observations 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 m^7GDP. The free T7 CAT RNA was mostly uncapped but, owing to the large amount, was translated albeit 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 m^7GDP. 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 think that they would be differentially capped.

DISCUSSION

In previous studies (1–3), the full potential of the vaccinia/T7 hybrid expression system was not realized because RNA made by T7 RNA polymerase is not as efficiently capped as RNAs made by the vaccinia virus polymerase. Since the cap is needed for both RNA stability and translation (22), this deficiency presented a severe challenge. The RNA stability problem was solved by retaining the T7 stem–loop segment at the 5′ ends of the transcripts (3). In considering ways of

improving translatability, the use of the UTR of certain RNA viruses seemed especially appropriate. RNAs of picornaviruses are efficiently translated in the absence of a cap apparently by a mechanism involving ribosome binding at an internal site within the UTR (8–11). Thus, we inserted a portion of the EMCV UTR between the T7 promoter and the CAT gene. Initial experiments indicated that the T7 stem–loop segment was still required, evidently for reasons of RNA stability as documented (3). Since the original T7 RNA start site and stem–loop segment were present at the 5′ ends of the transcripts, we assumed that the RNAs were still poorly capped. Nevertheless, expression increased markedly as judged by accumulated CAT activity, metabolic labeling of polypeptides, and visualization of the CAT protein in stained polyacrylamide gels. All of our data are consistent with a mechanism that involves cap-independent translation conferred by the EMCV UTR. These findings include (*i*) the cap-independent translatability in reticulocyte extracts of EMCV UTR-containing transcripts made *in vitro* from our expression plasmids and *in vivo* from recombinant viruses; (*ii*) the absence of an appreciable effect of the EMCV UTR on RNA accumulation in infected cells; (*iii*) the greater proportion of UTR-containing transcripts, relative to non-UTR-containing ones, in the polyribosome fraction; and (*iv*) the selective effect of hypertonic conditions on expression of the EMCV UTR-containing RNA *in vivo*. The latter result is compelling since it is well known that the translation of most cellular mRNAs is reduced under hypertonic conditions (23) and Carrasco and co-workers (20, 24) have proposed that increased intracellular sodium ion concentration contributes to the selective synthesis of viral proteins. Especially pertinent to our work is the report that elevated salt concentrations, sufficient to inhibit the translation of host cell mRNA, dramatically stimulate the translation of EMCV RNA (24). We noted that hypertonic conditions not only elevated expression of EMCV UTR CAT constructs but simultaneously decreased the expression of vaccinia virus late mRNA, which is known to be capped (25). It seems likely, therefore, that this differential salt effect is mediated via the postulated internal ribosome binding site of the EMCV UTR.

We had wondered previously whether the expression that did occur with the original vaccinia virus/T7 hybrid system resulted from efficient translation of the low amount of capped RNA or from inefficient translation of the excessive amounts of uncapped RNA (3). The present study suggests that the former was the case. The T7 transcripts that were isolated from the polyribosome fraction appeared to be enriched in capped species since their translation in reticulocyte extracts was inhibited by the cap analog m^7GDP, whereas translation of the free RNA was not inhibited appreciably.

The mammalian cell expression system described here 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. It may be used most simply by transfecting a plasmid with 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 to prepare a new recombinant vaccinia virus. Alternatively, by recombining the T7 promoter-regulated gene into vaccinia virus and using a double-infection protocol, larger numbers of cells may be conveniently used and still higher expression may be obtained. Model studies with the small (25-kDa) CAT protein revealed that it was the dominant polypeptide made in vaccinia virus-infected cells and after 24 hr accumulated to a level of 16.5 μg per 2×10^6 cells, which accounted for $\approx$10% of the 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 promoters described thus far are used (26, 27). The vaccinia hybrid system has other advantages, such as use for potentially toxic genes, since expression does not occur until cells are coninfected with the T7 RNA polymerase containing helper virus.

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