JOURNAL OF VIROLOGY, Mar. 1992, p. 1370–1376
0022-538X/92/031370-07$02.00/0

Vol. 66, No. 3

Tacaribe Arenavirus RNA Synthesis In Vitro Is Primer Dependent and Suggests an Unusual Model for the Initiation of Genome Replication

DOMINIQUE GARCIN AND DANIEL KOLAKOFSKY*

Department of Genetics and Microbiology, University of Geneva School of Medicine, CMU, 9 Avenue de Champel, CH1211 Geneva, Switzerland

Received 3 September 1991/Accepted 25 November 1991

A Tacaribe virus in vitro system for RNA synthesis was established and found in large part to faithfully reproduce RNA synthesis in vivo. Similar to influenza virus and bunyavirus in vitro systems, this system was also highly dependent on added oligonucleotides. Of the eight tested, only three were active, in the order GpC > CpG > ApApC. Determination of the 5′ ends of the transcripts suggested that the oligonucleotides were acting as primers. In particular, whereas stimulation with CpG (complementary to positions +1 and +2 of the template) led to RNAs whose 5′ ends were at position +1 as expected, GpC stimulation led to transcripts whose 5′ ends were at position −1 rather than at position +2, as GpC is complementary to positions +2 and +3 of the template. This finding suggests a model for the initiation of genome replication in which pppGpC is first made on the template at positions +2 and +3 but slips backwards on the template so that the 5′ end is at position −1 before elongation can continue.

Arenaviruses such as Tacaribe virus (TAC) contain bisegmented single-stranded RNA genomes called S (small) and L (large). Although TAC is similar in many respects to other segmented negative-strand RNA viruses such as bunyaviruses and influenza viruses, this terminology is inappropriate to describe the TAC genome, as both arenavirus segments employ an ambisense coding strategy (2, 4, 8, 21). By convention, the S and L chains found in virions are called simply genomes and their complements are called antigenomes, without reference to polarity based on protein coding (Fig. 1). mRNAs are transcribed from the 3′ ends of both genome and antigenome segments, and these segments terminate within an intergenic region which separates the coding regions of each segment (8). Those from the S and L genomes code for the nucleoprotein N and the large protein L, respectively, and those from the S and L antigenomes code for the glycoprotein precursor GPC and a small protein called Z or P11, respectively.

The manner in which arenavirus RNA synthesis is initiated has recently been investigated (6, 20). The mRNAs were found to contain from one to five extra nontemplated bases at their 5′ ends which are capped, and the extra bases are also heterogeneous in sequence. In this respect, TAC mRNA is similar to influenza virus (reviewed in references 12 to 14) and bunyavirus (reviewed in reference 11) mRNAs, which obtain these nontemplated bases from host mRNAs through a cap-snatching mechanism of initiation, except that the 5′ extensions of the latter viral mRNAs are both longer and more heterogeneous in length (10 to 18 bases long). It is possible that the 5′ extensions on arenavirus mRNAs are also derived from host mRNAs via cap snatching, but that much shorter fragments from host mRNAs would be taken as primers.

Remarkably, the 5′ ends of genomes and antigenomes also contained apparently nontemplated bases, except that in these cases there was only a single extra base, which was invariably G. These ends were not capped, however, and at least some of them started with (p)ppG (6). Genomes and antigenomes may therefore also start on a primer, but one that is not capped and of defined sequence. The minimum primer for TAC genome and antigenome synthesis would be pppGpC, and only the C residue would align with the G found at the 3′ ends of genome and antigenome templates. This primer could also result from a cytoplasmic host RNA by cleavage; however, the only such triphosphorylated RNA that we know of is 5S rRNA, which in eucaryotes starts with pppGpU. Alternatively, the pppGpC could be made by the viral polymerase, but not from the precise 3′ end of the template (6). To learn more about how arenavirus RNAs are initiated, we have examined RNA synthesis in vitro.

MATERIALS AND METHODS

Virus and cells. TAC-DA, obtained from David Auperin, Centers for Disease Control, Atlanta, Ga., was cloned in BHK-21 cells. A single plaque was passaged twice in BHK cells, and the medium was harvested at 48 h postinfection for virus stock.

Plasmids. A *ClaI-KpnI* fragment containing part of the polylinker of the pAT vector and nucleotides (nt) 1 to 439 of the TAC S genome from the strain of Franze-Fernandez (TAC-FF) (4) was cloned between the same sites of pBluescript KS to generate pTAC S 1-439.

A *DdeI-DdeI* fragment containing nt 1699 to 2149 of the TAC S genome, corresponding to the intergenic region, was blunt ended and cloned into the *SmaI* site of pGEM-4 to generate pTAC S 1699-2149.

To create plasmids for the generation of RNA markers that contain the TAC S sequence and whose 5′ ends are at position +1 or −1 relative to our strain (TAC-DA) for the experiment in Fig. 5, two fragments were amplified by polymerase chain reaction from pTAC 1-439, using as primers either 3′-CGGATCCTAGGTGACACGCGATATCACT CAGCATAA-5′, with a T7 promoter (underlined) fused to nt −1 to 19 of the TAC S antigenome with a single extra G

* Corresponding author.

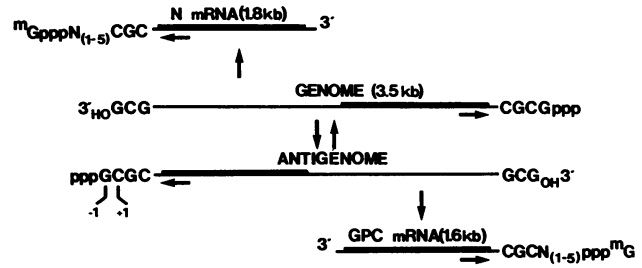

FIG. 1. Schematic representation of the TAC S-segment RNAs. RNAs are shown as horizontal lines, the thicker portions representing protein-coding sequences. The 5′ and 3′ ends are shown as triphosphates and hydroxyl groups, respectively. Vertical arrows show the direction of RNA synthesis. Short horizontal arrows show the locations of primers that can be used to determine the positions of the 5′ ends of the RNAs. Position +1, defined as the precise 3′ end of the template or its complement on the opposite strand, and position −1 are indicated.

(double underlined) at position −1, or 3′-CGGATCCTAG GTGACACGCGGATATCACTCAGCATAA-5′, with two extra G's (double underlined) at positions −1 and −2, and 3′-CCTTCACGGTTCGAAGTC-5′, representing positions 91 to 108 of the TAC S genome. The amplified fragments were then cloned into the *Sma*I site of pSP64 to generate pTAC SA -1-19 and pTAC SA -2-19. The positions refer to the 3′ end of the S genome (4, 20).

In vitro transcription system. Confluent cultures of BHK cells were infected with a 1:200 dilution of virus stock and maintained in minimal essential medium plus 2% fetal bovine serum at 33°C for 72 h. This culture medium was then used undiluted to infect other cultures, to obtain the highest possible multiplicity of infection. At 48 h, the cells were harvested by scraping into phosphate-buffered saline and recovered by centrifugation. At this step, intracellular RNAs could be separated into band and pellet fractions on CsCl density gradients as described by Raju et al. (20).

The infected cells were resuspended briefly (ca. 30 s) in a buffer containing 5% sucrose, 80 mM KCl, 35 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 5 mM K_2PO_4, 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 200 μg of lysolecithin per ml at 4°C and then repelleted (5 min at 1,000 rpm at 4°C). Cells from three 9-cm dishes were resuspended into 150 μl of buffer containing 200 mM KCl, 100 mM Tris (pH 7.5), 10 mM magnesium acetate, and 4 mM dithiothreitol. The cells were disrupted by pipetting 30 times and then centrifuged at 4,000 rpm at 4°C for 10 min to remove nuclei and cell debris. The resultant cytoplasmic extract was used for in vitro RNA synthesis in a total volume of 300 μl containing 150 μl of lysolecithin extract, 40 U of creatine phosphokinase, 10 mM creatine phosphate, 200 ng of actinomycin D, 1 mM spermidine, 500 μM each ATP, CTP, and GTP, 100 μCi of [α-^{32}P]UTP, and 30 μl of rabbit reticulocyte lysate (Promega). Samples were incubated at 30°C for 2 h, followed by RNA separation into band and pellet fractions on CsCl density gradients.

RNase protection assay. Cold or nonradioactive riboprobes were purified on sequencing gels, using the same radioactive riboprobe as a marker. The radiolabelled RNA made in vitro was hybridized with 50 to 100 ng of cold riboprobe (1:20 of a T7 reaction) in 50 μl of 2.5× buffer A (375 mM NaCl, 25 mM Tris-Cl [pH 7.4], 2.5 mM EDTA). The mixture was incubated at 85°C for 15 min and then at 65°C for 3 h. Then 200 μl of 2.5× buffer A containing 20 μg of RNase A and 10 U of RNase T_1 was added, and the mixture was incubated at 30°C for 1 h. The RNase was then digested with 150 μg of proteinase K per ml in the presence of 0.5% sodium dodecyl sulfate for 15 min at 37°C. After phenol-chloroform extraction and ethanol precipitation, the remaining RNAs were electrophoresed on a 6% polyacrylamide gel containing 7 M urea.

5′ end determination of the RNAs made in vitro. Radiolabelled RNAs made in vitro with the TAC polymerase extract, or the marker RNAs made with T7 polymerase, were combined with 20 pmol of cold primer (corresponding to positions 73 to 92 of the TAC S genome) in 2.5 M ammonium acetate and ethanol precipitated. Cold primer extension reactions were performed as previously described (20) except that a final volume of 50 μl and RNase H^- reverse transcriptase (Bethesda Research Laboratories) were used. Then 200 μl of RNase digestion buffer containing 2.5× buffer A with RNases A and T_1 was added, and the assay was continued as described above for RNase protection.

RESULTS

Characterization of the in vitro system. To examine both genome replication and transcription if possible, we used cytoplasmic extracts of infected cells rather than purified virions. Moreover, an in vitro system in which lymphocytic choriomeningitis virus RNA was synthesized from intracellular nucleocapsids (NCs) had previously been reported (5). To examine the de novo-made RNAs, the reaction mixtures were labelled with [^{32}P]UTP. We found, however, that TAC RNAs accounted for only a small fraction of the label incorporated, using both whole cytoplasmic extracts and their NCs obtained by centrifugation, even in the presence of actinomycin D (not shown; 5). In addition, viral RNA synthesis was stimulated by the addition of a rabbit reticulocyte lysate, which also contained endogenous UTP incorporation activity. Viral RNA synthesis was therefore determined by RNase protection after annealing with nonradioactive viral riboprobes and after separation of the reaction products on CsCl density gradients into NC RNA (which bands at 1.31 g/ml) and the pelleted unencapsidated RNA. We have previously shown that in vivo, the viral mRNAs are predominantly found in the pellet fraction and the genomes and antigenomes are predominantly found in the CsCl band fraction of these gradients (20).

Figure 2 shows the results of protecting the pellet RNA of various in vitro reactions with a cold probe complementary to nt 1 to 438 of the S antigenome and the N mRNA. This riboprobe was chosen first because the N mRNA is the predominant S antigenic-sense RNA and should be present mostly in the CsCl pellet fraction. The negative control here is a reaction with uninfected cell extract (lane Un.), and the positive control is a classical RNase protection assay in which the probe is radioactive and protected with cold infected cell RNA (lane Control). Lane In. shows that when infected cell extract is used, a weak band of the expected size (438 nt) can be detected, representing de novo RNA synthesis. This RNA synthesis was strongly stimulated by the addition of GpC (250 μM) to the reaction mixture, but the addition of GpU had no effect. Addition of GpC to uninfected cell extracts had no effect (not shown).

To examine the 3′ end of the antigenomic-sense RNA made in vitro, the CsCl pellet RNA was protected with a probe that has the same polarity but spans the intergenic region (Fig. 3). To determine the position of the 3′ end(s) of intracellular N mRNA in this assay, nonradioactive CsCl

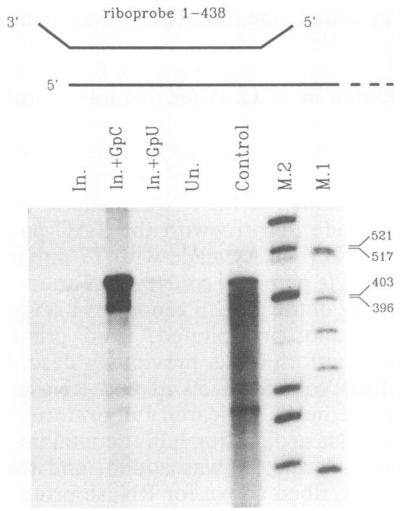

FIG. 2. Promoter-proximal RNA synthesis. A diagram of the RNase protection experiment is shown at the top; the riboprobe represents genomic-sense RNA. TAC polymerase reactions were carried out with either infected (In.) or uninfected (Un.) cell extracts, with or without the addition of 250 μM GpC or GpU, as indicated above the lanes. The levels of the radiolabelled transcripts made which pelleted through a CsCl density gradient were estimated by RNase protection using a cold riboprobe as described in Materials and Methods. Lane Control shows the band obtained when a radioactive riboprobe and cold intracellular CsCl pellet RNA was used. M.1 and M.2 refer to DNA length markers; some of their lengths are shown at the right in nucleotides.

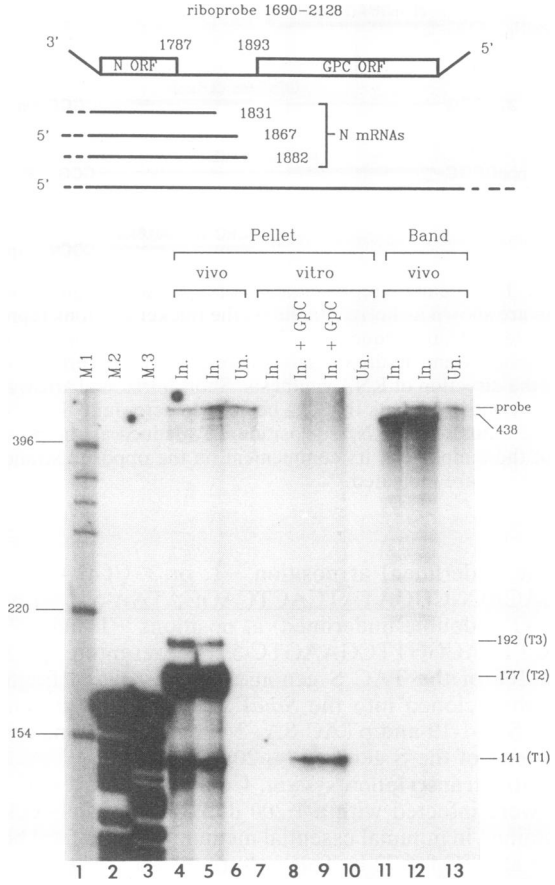

FIG. 3. Antigenomic-sense RNA synthesis near the intergenic region. Radiolabelled polymerase reactions were carried out (lanes 7 to 10), and the products were separated into CsCl band and pellet material. GpC was added to some reaction mixtures at 250 and 750 μM (lanes 8 and 9, respectively). The levels of the N mRNAs made and the positions of their 3′ ends were determined by using CsCl pellet material and a cold riboprobe as diagrammed at the top. Numbers refer to nucleotide positions of the S-genome segment. Positions of the 3′ ends of intracellular N mRNAs were determined by using cold CsCl pellet RNA from infected cells (lanes 4 and 5) and are indicated at the right. T1, T2, and T3, refer to mRNA termination sites. A marker band for S antigenome RNA was created by annealing intracellular CsCl band RNA to the radiolabelled riboprobe (lanes 11 and 12). Lanes M.1, M.2, and M.3 represent DNA restriction fragment markers and two lanes of a sequence ladder, respectively; sizes are shown at the left in nucleotides.

pellet RNA was used to protect the same probe that had been radiolabelled. As shown in lanes 4 and 5 of Fig. 3, there is one major 3′ end (T2, at ca. position 1867) and two minor ones (T1 and T3, at ca. positions 1831 and 1882), all within the intergenic region. There are two possible stem-loop structures in the S intergenic region (8), and T1 and T2 would map to the downstream side of the first and second structures, respectively; T3 would be downstream of the second structure. The CsCl pellet RNA made in vitro had extended to the intergenic region and appeared to end exclusively at the earliest termination site used in vivo (position 1831 or T1; lane 7). This RNA synthesis was again strongly stimulated by the addition of 250 μM GpC (lane 8), and further addition of GpC (to 750 μM) had little effect (lane 9). When the CsCl band RNA was examined, we were unable to detect a clear signal, even though S antigenomes were detectable in classical RNase protection assays using nonradioactive CsCl band RNA (lanes 11 and 12). However, S antigenomes would be expected to be made much less abundantly than N mRNAs, and there is the added complication of measuring antigenomes in the presence of an excess of genomes with this method.

As S genomes are about 5- to 10-fold more abundant than antigenomes in vivo, the in vitro-made RNAs were examined with a probe to the same region but complementary to S genomes and the GPC mRNA. The 3′ end of intracellular GPC mRNA was again determined by classical RNase protection with nonradioactive CsCl pellet RNA (Fig. 4, lane 2). A major band of 320 nt was found which would place the 3′ end roughly at position 1808 (T2), and a minor one (280 nt) was found a little earlier, at position 1848 (T1), again both within the intergenic region and mapping to the downstream side of the two stem-loop structures (8). There also appears to be some genome RNA within the CsCl pellet, as evidenced by a band of the same size as the viral sequences of the probe (438 nt), which is the predominant band when intracellular NC RNA is used to protect the probe (lane 1). Lanes 4 and 5 show that the RNA made in vitro which pelleted through the CsCl gradient had again terminated mostly at the first site used in vivo and was again strongly stimulated by the presence of GpC. Addition of cycloheximide to this reaction mixture had no effect (lane 6), indicating that RNA synthesis was independent of protein synthesis despite stimulation by the rabbit reticulocyte lysate. Examination of the CsCl band RNA showed that read-through RNAs indicative of genomes were made in vitro; however, these were stimulated only slightly (ca. twofold) with GpC, and the addition of cycloheximide only slightly reduced the

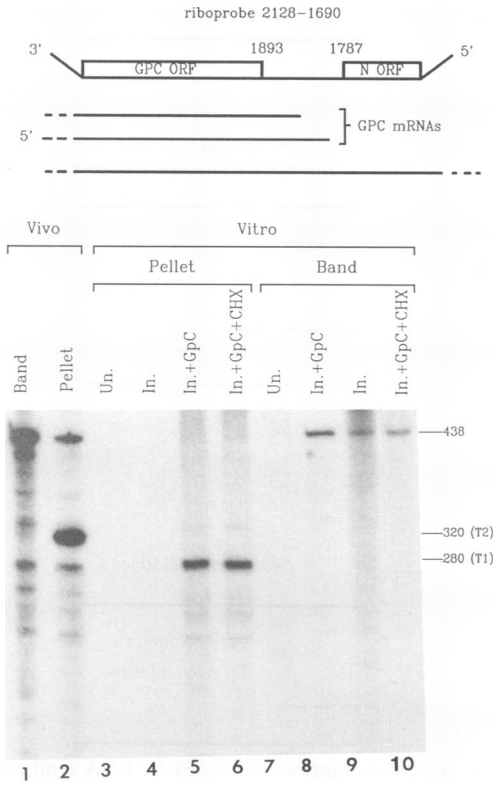

FIG. 4. Genomic-sense RNA synthesis near the intergenic region. The experiment was as described for Fig. 3 except that the riboprobe was complementary to S genomes and GPC mRNA, and in vitro-made CsCl band RNA was also examined. T1 and T2 refer to the two GPC mRNA termination sites used in vivo. The experiment is diagrammed at the top. CHX, cycloheximide.

level of genome synthesis. Presumably, the encapsidation of the genome RNAs made in vitro is due mostly to a preexisting pool of N protein within the cell extract.

This in vitro system therefore appears in large part to reflect viral RNA synthesis in vivo. Both N and GPC mRNA-like transcripts which terminate at the earliest of the sites used in vivo are made and are found predominantly in an unencapsidated form (CsCl pellet fraction). Genome-sense RNAs which have read through the intergenic region are also made, and these are found predominantly in the encapsidated region of the gradient. Moreover, TAC RNA synthesis in vitro is strongly dependent on the addition of GpC to the reaction mixture.

The 5′ ends of the RNA made in vitro. As mentioned above, arenaviruses are different from other RNA viruses in that the 5′ ends of their genomes and antigenomes contain one extra G (Fig. 1). We have previously suggested (6) that this apparently nontemplated G could result from an oligonucleotide such as GpC (or pppGpC) being used as a primer for genome replication. Moreover, of the six dinucleotides (GpC, GpA, GpU, CpG, CpU, and UpC) and two trinucleotides (ApApC and ApCpC) that we tested, only GpC, CpG, and ApApC stimulated the reaction, and of these three, GpC clearly had the strongest effect (data not shown and Table 1). However, it is important to determine whether these oligonucleotides stimulate the reaction by acting as primers. If so, one might expect that the 5′ ends of the resulting transcripts would be at different positions.

TABLE 1. Stimulatory effects of oligonucleotides on TAC RNA synthesis in vitro[a]

Band position	No addition	Level of product				
		+ApApC	+GpC	+CpG	+GpU	+CpU
−4	1 ± 0.1	1.33 ± 0.4	1.69 ± 0.5	1.77 ± 0.5	1.18	0.59 ± 0.2
−2		10.33 ± 1.5				
−1	1		68.9 ± 11		1.38	0.55 ± 0.1
+1				27 ± 1		

[a] Various oligonucleotides (500 μM) were used to stimulate the polymerase reaction. The products were examined as described for Fig. 5, and their levels were determined in a Phosphoimager. In all cases, the level at position −4 was determined. For the stimulated reactions, only the longest and strongest band was determined. Values are averages and ranges of two separate experiments, normalized by setting the band at −1 in the unstimulated reactions to a value of 1.

The classical procedure for determining the 5′ ends of RNAs is to use primer extension. However, as the de novo-made RNAs represent only a small fraction of the endogenous RNAs in the extract, only the RNAs labelled in vitro could be examined. The following protocol was therefore adopted (Fig. 5). A nonradiolabelled primer extension reaction was carried out with RNase H^- reverse transcriptase on the labelled RNAs made in vitro. Then the portion of the in vitro transcript protected against RNase digestion by the nonradioactive cDNA was isolated and

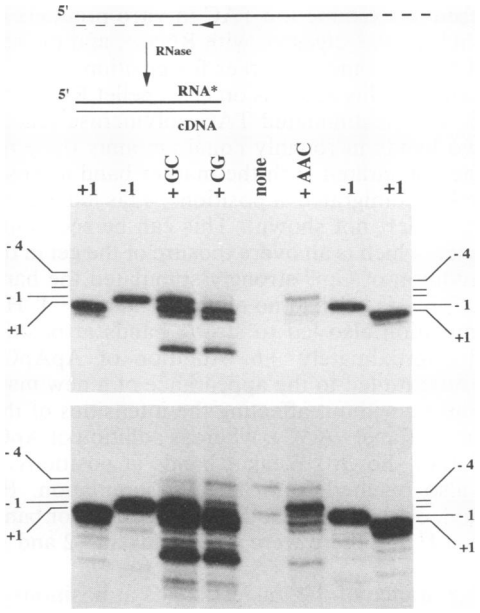

FIG. 5. Mapping the 5′ ends of the reaction products stimulated with GpC, CpG, and ApApC. Radiolabelled CsCl pellet RNA from either unstimulated reactions (lane none) or those stimulated with either GpC, CpG, or ApApC (indicated above the lanes) were extended with $RNase^-$ reverse transcriptase and a primer complementary to nt 73 to 92 of the S antigenome. The resulting RNA-cDNA hybrids were then digested with RNase, and the protected fragment was run on a 6% sequencing gel. Lanes +1 and −1 refer to marker RNAs whose 5′ ends are at these positions, generated as described in the text. The positions of the 5′ ends are shown at the sides. The two panels show different exposure times. The experiment is diagrammed at the top; the horizontal arrow represents the primer, and the asterisk indicates the radiolabelled strand.

separated on a sequencing gel. To generate precise markers for this experiment, it is necessary to have RNAs, rather than DNAs, of the relevant viral sequences whose 5′ ends are at positions +1 and −1. These can normally be made in vitro with T7 polymerase by using plasmids in which the viral sequences are placed just downstream of the T7 promoter. For the marker RNA whose 5′ end is at position +1, this would require placing the sequence 5′-CGCA. . . next to the promoter. Unfortunately, under these conditions, T7 polymerase starts the chain at position +2 with GTP, as it has a strong aversion to starting chains with CTP (16; data not shown). Serendipitously, we noticed that the strain of TAC used by Franze-Fernandez et al. (TAC-FF) (4) and our strain (TAC-DA) are identical within the first 120 nt except that TAC-FF contains a run of 6 U residues at position 47 to 52, whereas TAC-DA contains a run of 7 (determined by directly sequencing the viral RNAs from each strain as well as by sequencing DNA clones [not shown]). We therefore placed the sequence 5′-GCGCA. . . next to the promoter, using DNA from TAC-FF. As expected, this construct started with GTP just after the promoter and made a transcript with one extra 5′ G but one less U in the U run relative to our virus, i.e., a marker RNA whose 5′ end is at position +1 for our virus in this protocol. A marker RNA whose 5′ end is at position −1 was then created by placing two extra G's, or the sequence 5′-GGCGCA. . ., next to the promoter. The 5′ ends of the marker RNAs were confirmed by classical primer extension on the T7 transcripts, using a sequence ladder generated with the same primer on these plasmid DNAs. A nonradioactive primer extension reaction was then carried out on the labelled T7 transcripts with the same primer used to examine the TAC in vitro products (nt 73 to 92), the hybrid was digested with RNase, and the remaining fragment then became a marker for position −1 or +1.

The results of this analysis on CsCl pellet RNA are shown in Fig. 5. The unstimulated TAC polymerase reaction produced two bands in roughly equal amounts (lane none), of which one comigrated with the marker band at position −1 and the other comigrated at position −4 (as judged by a DNA sequence ladder; not shown). This can be seen only in the lower panel, which is an overexposure of the gel in the upper panel. Addition of GpC strongly stimulated the band at −1 (68-fold; Table 1) but had no effect on that at −4. However, GpC stimulation also led to strong bands at positions +1, +2, and approximately +6. Addition of ApApC to the reaction mixture led to the appearance of a new major band at position −2 without affecting the intensities of the bands at −1 and −4 (lane +AAC), whereas addition of ApCpC had no effect (not shown). Weaker bands at positions +1, +2, and +6 also resulted from ApApC stimulation. For CpG stimulation, on the other hand, the longest major band was at positions +1, and there were also bands at +2 and +6 (lane +CG).

The appearance of the longest bands at positions −2 with ApApC, −1 with GpC, and +1 with CpG strongly suggests that these oligonucleotides were acting as primers, as depicted in Fig. 6b, at least to some extent. It would be difficult to explain the pattern of the longest bands which resulted otherwise. It is possible that the appearance of the bands at +2 and +6 in all cases and the band at +1 for ApApC and GpC is due to the ability of these oligonucleotides to stimulate the polymerase to start at these positions without acting as primers. However, it is also possible that these shorter bands are specific degradation products, as overexposure of the gel shows similar bands in some of the marker RNA lanes.

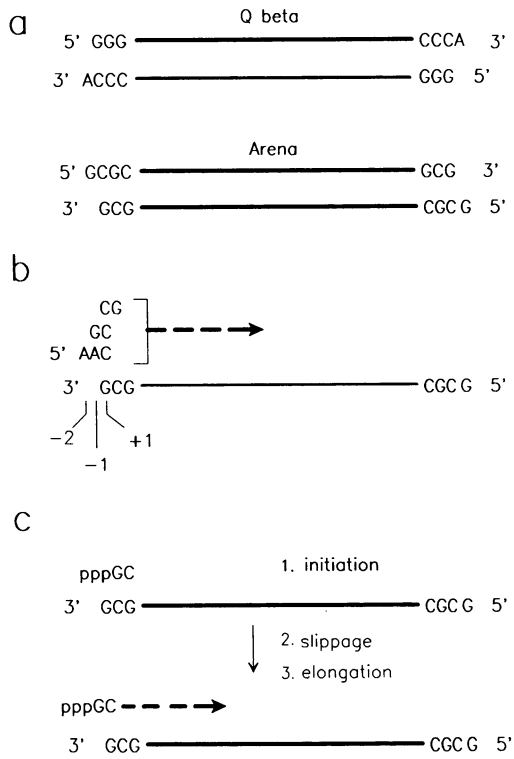

FIG. 6. Schematic representation of TAC RNA synthesis in vitro and a model for the initiation of genome replication in vivo. (a) Alignment of bacteriophage Qβ and arenavirus genomes and antigenomes. Only the nucleotides at the ends are shown. In both cases there is a single base overhang, but on opposite strands. (b) Model for how oligonucleotides act as primers for TAC RNA synthesis in vitro and create transcripts whose 5′ ends are at different positions, as determined in these studies. The precise 3′ end of the template is referred to as position +1; extra bases on the complementary 5′ end are given minus designations. (c) Model for how genomes and antigenomes with 5′ ends at position −1 might be created in vivo, including the generation of the primer.

In other experiments (not shown), when the RNA-cDNA hybrids were immunoselected with an anticap antiserum before RNase digestion (7a), only the band at −4 was selected in each reaction, similar to what is found in vivo (6). One difference between the capped 5′ ends of the mRNAs found in vivo and those made in vitro is that positions −3 to −5 are clearly seen in vivo, whereas only position −4 is seen in vitro. It is possible that the unique band at −4 in vitro is due to globin mRNA acting as the source of the primer. If so, the natural mechanism of mRNA initiation (presumably cap snatching) would also appear to take place in vitro. Moreover, examination of the CsCl band RNA in these experiments showed that <10% of the products were encapsidated and that the band at −4 was underrepresented, the latter as expected from in vivo studies. Unexpectedly, however, we found that chains which began at positions −2 (addition of AAC) or +1 (addition of CG) were as well represented in these experiments as those that started at −1, which is the only 5′ end encapsidated in vivo. It would appear that the precise positions and sequences of the 5′ ends of the transcripts have little effect on whether they will be encapsidated, at least in vitro, but that the presence of a cap group may play a role.

The arenavirus polymerase, like that of influenza viruses

and bunyaviruses, can thus also be stimulated by primers in vitro. More importantly, whereas the minimum dinucleotide primer (ApG) which stimulates the influenza virus and bunyavirus polymerases leads to transcripts which start at position +1 (17, 19). GpC, which best stimulates the TAC polymerase, yields RNAs which start at −1.

DISCUSSION

Because of the unusual 5′ ends of the TAC RNAs found in vivo, we have examined viral RNA synthesis in vitro. Using NCs from lymphocytic choriomeningitis virus-infected cells, Fuller-Pace and Southern (5) had previously reported that genomes as well as mRNAs were made. We found this surprising, as their NCs were treated with high salt and detergent before being pelleted through a 50% sucrose cushion and so should be free of a soluble pool of N protein thought to be required to support genome replication. This result raised the possibility that genomes could be made without concurrent assembly of the chains. However, all of the TAC genome RNAs made in vitro in this study were found in a completely encapsidated form (Fig. 4). Although these experiments were carried out with crude extracts, we have also used pelleted NCs and obtained precisely the same result (not shown). It is possible that the pool of N required to encapsidate the arenavirus genomes made in vitro is not in a soluble form.

The relationship between the viral polymerases which carry out transcription and replication is not entirely clear, but they presumably initiate their chains differently, and mRNA synthesis is not coupled to chain assembly. In most in vitro systems with negative-strand RNA viruses described to date, it has been difficult to detect much genome synthesis, and this is also so in the studies presented here. Encapsidated transcripts which had read through the intergenic region (i.e., genomes) represented <10% of the products; >90% of the products pelleted through the CsCl gradient, and their 3′ ends mapped within the intergenic region like those of in vivo mRNAs (whereas their 5′ ends resembled those of genomes). Moreover, the synthesis of transcripts which ended within the intergenic region was strongly stimulated by GpC, but genome synthesis was stimulated only slightly. Genome synthesis in these systems is presumably limited by the requirement for concurrent assembly, which operates poorly. As the initiation of assembly is likely to be the rate-limiting step in this process, most of the genome synthesis that we do find may very well be the result of elongating preinitiated replicative intermediates, hence the very modest stimulation of genome synthesis by GpC. In such an in vitro system, once the transcripts have been initiated by artificially high levels of GpC, the products can be expected to be mostly mRNA-like regardless of whether a transcriptive or replicative polymerase initiated these chains. One cannot assume that only transcriptases were active simply because the products formed 3′ ends like mRNAs. If so, the information drawn from these studies would also apply to genome replication. The finding that GpC not only is the most efficient primer in these assays but also creates 5′ ends which mimic those of genomes suggests that these studies are in fact relevant to the initiation of genome chains.

According to the studies described here, in vitro RNA synthesis with all segmented negative-strand RNA viruses is highly dependent on oligonucleotide priming. For influenza viruses and bunyaviruses, the requirement for ApG is thought to be simply a way of bypassing the natural route of chain initiation, i.e., cap snatching (mRNA) or initiation with pppA (genome replication). Most RNA polymerases will initiate with dinucleotides representing the start sequence instead of nucleoside triphosphates (3). For influenza virus, only 3 of the 16 possible NpNs were found to be active, in the order ApG > GpG >> GpC (15, 18). There is evidence that ApG primes influenza virus RNA synthesis at the precise 3′ end of the template (3′-UCG. . .) to which it is complementary (19). We assume that GpG acts similarly to ApG except that the first base pair would be G · U rather than A · U and that GpC acts by pairing with positions +2 and +3 of the template, consistent with their order of activity. The 3′ end of arenavirus templates is similar but is 3′-GCG. . . . If the processes of initiation of arenavirus and influenza virus RNA synthesis were mechanistically identical (as appears to be so for influenza viruses and bunyaviruses [11]), one would have expected CpG to be more active than GpC and to have initiated chains at positions +1 and +2, respectively. However, whereas CpG initiates at +1 as expected, GpC initiates chains at position −1 rather than +2 and is more active than CpG. We assume that ApApC is the least active because it can form only one base pair with the 3′ end of the template (Fig. 6b) and cannot first anneal internally like GpC.

One possible explanation for these results is shown in Fig. 6c. The 5′ end of arenavirus genomes and antigenomes appears to be the nontemplated (p)ppG (6). We assume that this viral polymerase, like all others known to date, cannot initiate with a pyrimidine triphosphate (1) and so initiates with GTP at position +2, in analogy with Qβ RNA polymerase (23). However, when the first phosphodiester bond is formed, the pppGpC would slip backwards on the template, realigning to create a chain whose 5′ end is position −1, before the polymerase can continue downstream. There is evidence that paramyxovirus mRNA editing occurs via similar controlled slippage events, but during elongation rather than initiation (22), and other RNA polymerases are known to slip during initiation at certain promoters (reviewed in reference 10). In this scheme, the extra 5′ G is not nontemplated but rather represents another example of pseudotemplated synthesis (10). GpC is presumably the most potent stimulator because it follows the proposed natural route. It first aligns on the template at positions +2 and +3, to which it is complementary, before slipping backwards by two bases such that only the 3′ C is aligned with the 3′ G of the template, hence the order of activity (GpC > CpG > ApApC) as well as the positions of the 5′ ends in the oligonucleotide-stimulated reactions.

Although the explanation presented above is not unreasonable, the rationale for such a mechanism seems obscure, and there is the added problem of how the overhanging ends are maintained. This latter problem is not unlike that of the telomeres of chromosomes. Because DNA synthesis begins on a RNA primer which is then removed, the 3′ ends of chromosome would get progressively shorter with continued replication if some corrective mechanism did not exist. Hence, one finds the continual growth of the 3′ ends due to a telomerase (7). A closer example is that of bacteriophage Qβ, for which the opposite but remarkably symmetrical situation applies (23) (Fig. 6a). During replication, a nontemplated A residue is added to the 3′ ends, presumably because this polymerase cannot simply run off the end of its template, and adds the A in the act of terminating synthesis. Again, if some counteracting mechanism did not exist, the ends of the genomes would grow. The mechanism for Qβ is to initiate chains not at the precise 3′ end of the template with UTP but

at the penultimate C with GTP, presumably because viral polymerases can initiate only with ATP or GTP. With this example in mind, it is possible that arenavirus polymerases can terminate chains only by removing the last base. The curious mechanism that we propose for how arenaviruses create the extra 5′ G would then be part of the mechanism to prevent the natural ends of the genome from getting shorter with continued replication.

ACKNOWLEDGMENTS

We thank Maria-Theresa Franze-Fernandez (Buenos Aires) for the restriction fragments from her clone of the TAC S segment used to create pTAC S 1-439 and 1699-2149.

This work was supported by a grant from the Swiss National Science Fund.

REFERENCES

1. **Banerjee, A. K.** 1980. 5′ terminal cap structure in eucaryotic messenger ribonucleic acids. Microbiol. Rev. **44**:175–205.
2. **Bishop, D. H. L., and D. D. Auperin.** 1987. Arenavirus gene structure and organization. Curr. Top. Microbiol. Immunol. **133**:5–17.
3. **Downey, K. M., B. S. Jurmark, and A. G. So.** 1971. Determination of nucleotide sequences at promoter regions by the use of dinucleotides. Biochemistry **10**:4970–4975.
4. **Franze-Fernandez, M. T., C. Zetina, S. Iapalucci, M. A. Lucero, C. Bouissou, R. Lopez, O. Rey, M. Daheli, G. N. Cohen, and M. M. Zakin.** 1987. Molecular structure and early events in the replication of Tacaribe arenavirus S RNA. Virus Res. **7**:309–324.
5. **Fuller-Pace, F. V., and P. J. Southern.** 1989. Detection of virus-specific RNA-dependent polymerase activity in extracts from cells infected with lymphocytic choriomeningitis virus: in vitro synthesis of full-length viral RNA species. J. Virol. **63**: 1938–1944.
6. **Garcin, D., and D. Kolakofsky.** 1990. A novel mechanism for the initiation of Tacaribe arenavirus genome replication. J. Virol. **64**:6196–6203.
7. **Greider, C. W., and E. H. Blackburn.** 1989. A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. Nature (London) **337**:331–337.

7a. **Hacker, D., S. Rochat, and D. Kolakofsky.** 1990. Anti-mRNAs in Lacrosse bunyavirus-infected cells. J. Virol. **64**:5051–5057.

8. **Iapalucci, S., N. López, and M. T. Franze-Fernandez.** 1991. The 3′ end termini of the Tacaribe arenavirus subgenomic RNAs. Virology **182**:269–278.
9. **Iapalucci, S., N. Lopez, O. Rey, M. M. Zakin, G. N. Cohen, and M. T. Franze-Fernandez.** 1978. The 5′ region of Tacaribe virus L RNA encodes a protein with a potential metal binding domain. Virology **173**:357–361.
10. **Jacques, J. P., and D. Kolakofsky.** 1991. Pseudo-templated transcription in prokaryotic and eukaryotic organisms. Genes Dev. **5**:707–713.
11. **Kolakofsky, D., and D. Hacker.** 1991. Bunyavirus RNA synthesis: genome transcription and replication. Curr. Top. Microbiol. Immunol. **169**:143–157.
12. **Krug, R. M.** 1981. Priming of influenza viral RNA transcription by capped heterologous RNAs. Curr. Top. Microbiol. Immunol. **93**:125–149.
13. **Krug, R. M.** 1983. Transcription and replication of influenza viruses, p. 70–98. *In* P. Palese and D. W. Kingsbury (ed.), Genetics of influenza viruses. Springer-Verlag, Vienna.
14. **Lamb, R. A., and P. W. Choppin.** 1983. The gene structure and replication of influenza virus. Annu. Rev. Biochem. **52**:467–506.
15. **McGeoch, D., and N. Kitron.** 1975. Influenza virion RNA-dependent RNA polymerase: stimulation by guanosine and related compounds. J. Virol. **4**:686–695.
16. **Milligan, J. F., D. R. Groebe, G. W. Witherell, and O. C. Uhlenbeck.** 1987. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. Nucleic Acids Res. **15**:8783–8798.
17. **Patterson, J. L., B. Holloway, and D. Kolakofsky.** 1984. La Crosse virions contain a primer-stimulated RNA polymerase and a methylated cap-dependent endonuclease. J. Virol. **52**:215–222.
18. **Plotch, S. J., and R. M. Krug.** 1977. Influenza virion transcriptase: synthesis in vitro of large, polyadenylic acid-containing complementary RNA. J. Virol. **1**:24–34.
19. **Plotch, S. J., and R. M. Krug.** 1978. Segments of influenza virus complementary RNA synthesized in vitro. J. Virol. **2**:579–586.
20. **Raju, R., L. Raju, D. Hacker, D. Garcin, R. Compans, and D. Kolakofsky.** 1990. Nontemplated bases at the 5′ ends of Tacaribe virus mRNAs. Virology **174**:53–59.
21. **Salvato, M. S., and E. M. Shimomaye.** 1989. The completed sequence of lymphocytic choriomeningitis virus reveals a unique RNA structure and a gene for a zinc finger protein. Virology **173**:1–10.
22. **Vidal, S., J. Curran, and D. Kolakofsky.** 1990. A stuttering model for paramyxovirus P mRNA editing. EMBO J. **9**:2017–2022.
23. **Weber, H., and C. Weissmann.** 1970. The 3′-termini of bacteriophage Qbeta plus and minus strands. J. Mol. Biol. **51**:215–224.