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NOTES

La Crosse Virus Small Genome mRNA Is Made in the Cytoplasm

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The cellular site of La Crosse virus S genome mRNA synthesis was examined by pulse-labeling infected cells for various times and determining the amount of labeled S mRNA in both the cytoplasmic and nuclear fractions. With pulse times as short as 3 min, La Crosse virus S genome transcription was found to be localized in the cytoplasm.

La Crosse virus (LaC) is a member of the California encephalitis serogroup of the insect-transmitted bunyavirus family (1). The genome of bunyaviruses consists of three segments of single-stranded RNA of negative polarity (−), each contained within separate nucleocapsids which are labeled small (S), medium (M), and large (L).Bunyaviruses are thus referred to as segmented (−) RNA viruses, a term which also includes the influenza virus and arenavirus families.

Bunyavirus S-genome mRNAs have recently been shown to contain 10 to 15 heterogenous, nontemplated nucleotides at their 5′ ends (2, 10). These nontemplated nucleotides are presumably the result of a cellular primer used to initiate transcription, similar to the situation demonstrated for influenza virus mRNAs (7). These findings suggested that purified LaC virions should thus also contain a primer-stimulated RNA polymerase; indeed further studies demonstrated that LaC transcription in vitro with purified virions could be stimulated by oligonucleotides such as $(A)_nG$, cap analogs such as $^mGpppA_m$, and natural mRNAs such as Alfalfa mosaic virus RNA 4 (9). Further, in the case of the (A)nG- and alfalfa mosaic virus RNA 4-stimulated reactions, the reaction products could be shown to contain the expected nontemplated extensions at their 5′ ends, suggesting that these molecules were indeed acting as primers for transcription. Purified LaC virions were also shown to contain an endonuclease which cleaves alfalfa mosaic virus RNA 4 only when this RNA carries a 7-methylated cap group, to produce capped fragments which are 13 and 14 nucleotides long, a length consistent with their action as transcription primers (9).

Interestingly, all of the above findings have previously been demonstrated as characteristics of the influenza virus polymerase (7). These remarkable similarities between the LaC and influenza virus polymerases and the mechanism by which they initiate their mRNAs prompted us to further compare their transcription. Two other features which are characteristic of influenza virus transcription, and which differentiate it from that of other (−) RNA viruses such as vesicular stomatitis virus, are that it takes place in the nucleus of the infected cell and that it is dependent on ongoing cellular mRNA synthesis (reviewed recently in reference 6). This latter characteristic has generally been interpreted as a requirement for a nascent or a newly synthesized mRNA chain as a substrate for the influenza virus endonuclease. The cellular location of bunyavirus transcription has so far not been examined directly, but this question can also be addressed by asking whether bunyaviruses can replicate in enucleated cells. Two groups have reported such experiments. Goldman et al. (4) found that California encephalitis virus could produce progeny in enucleated XC cells; however these experiments did not contain a control virus, such as influenza virus, which cannot replicate in enucleated cells. Pennington and Pringle (11), on the other hand, found that bunyamwera virus did not produce progeny in enucleated BHK cells under conditions in which vesicular stomatitis virus progeny formation was unaffected. These apparently conflicting results thus render interpretation difficult.

In contrast to influenza virus transcription, LaC transcription is known to be independent of cellular mRNA synthesis (reviewed in reference 8). An example of this independence is shown in Fig. 1, where oligodeoxynucleotide primer extension has been used to determine both the amount of LaC S-mRNA and the position of its 5′ end of RNA made in vivo either in the presence or absence of actinomycin D (Act D). This experiment demonstrates that the addition of Act D to LaC-infected cells at either 2 or 4 h postinfection (p.i.) actually increases the steady-state concentration of S mRNA 2 h later. Further, the S mRNA made in the absence of ongoing cellular mRNA synthesis contains the same heterogeneous distribution of 5′ ends as the S mRNA made under control conditions, i.e., the majority of S mRNAs contain 5′ extentions which are 10 to 15 nucleotides long and were thus presumably initiated by the same cellular primers. This experiment would argue that the LaC polymerase uses a stable pool of mRNAs as a source of primers and hence that LaC transcription should be localized in the cytoplasm.

The above experiment is, however, a rather indirect way of localizing the cellular site of LaC transcription. To more directly examine this point, LaC-infected cells were pulse-labeled with [^{3}H]uridine for short periods of time and then fractionated into nuclear and cytoplasmic fractions as fol-

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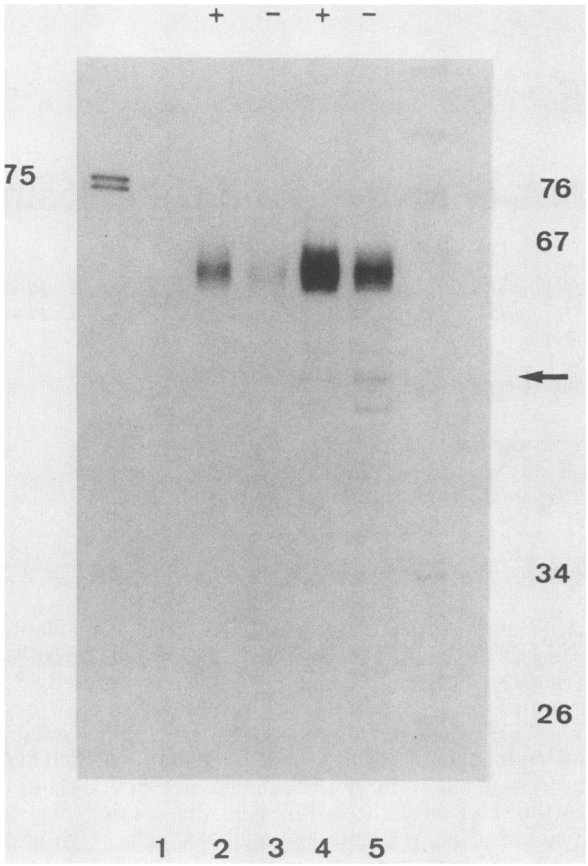

FIG. 1. Effect of Act D on the intracellular concentration of LaC S mRNA. Replicate cultures of BHK cells were infected with LaC (multiplicity of infection, 20). Act D (1 μg/ml) was added to some of the cultures at 2 h p.i., but not to others, and the cultures were harvested at 4 h p.i. (lanes 2 and 3, respectively). Similarly, Act D was added to some cultures at 4 h p.i. but not to others, and these cultures were harvested at 6 h p.i. (lanes 4 and 5, respectively). CsCl pellet RNA was then prepared from these cultures plus from mock-infected cells (lane 1), and the amount of S mRNA in 2 optical density units of each sample was estimated by primer extension of an end-labeled oligodeoxynucleotide representing nucleotides 38 through 50 from the 3′ end of the LaC S (−) genome RNA, as previously described (9). The arrow on the right side shows position no. 1 or the exact 5′ end of the (+) antigenome (9). Plus and minus signs above the figure indicate the presence and absence of Act D, respectively.

lows. Replicate cultures of three 10-cm dishes of BHK cells were infected at a multiplicity of infection of 50. After 1 h at 33°C, the infecting medium was replaced with minimal essential medium containing 2% fetal bovine serum and 2 μg of Act D per ml. At 4.5 h p.i., the medium was changed to include 10 mM glucosamine, and at 5 h p.i. the cultures were pulse-labeled for either 3, 6, 15, or 30 min with 4, 2, 1, or 0.5 mCi of [^{3}H]uridine per dish. The cells were then harvested in phosphate-buffered saline, disrupted in 1% Nonidet P-40–100 mM NaCl–10 mM Tris chloride (pH 7.4)–1.5 mM $MgCl_2$, and centrifuged for 5 min at 1,000 × *g*. The pellet was then suspended in 0.3% Nonidet P-40 and 0.2% deoxycholate plus the above salts, vortexed, and centrifuged. The combind supernatants were then centrifuged on CsCl density gradients as previously described (10), whereas the nuclear pellet was suspended in a mixture of Sarcosyl, guanidinium-thiocyanate and β-mercaptoethanol, sheared in a miniblender, and centrifuged in a CsCl gradient of greater density, also as previously described (3). In this method, LaC mRNA from the cytoplasmic fraction was recovered free of encapsidated genome and antigenome RNA by pelleting through a CsCl density gradient (H. Lindsey-Regnary, Ph.D. thesis, Emory University, Atlanta, Ga., 1983), whereas for technical reasons total LaC RNA was recovered from the nuclear fraction by the Sarcosyl–guanidinium-SCN–CsCl density gradient procedure. The amount of pulse-labeled Lac S (+) RNA in each cellular fraction was then determined by annealing with an excess of LaC S (−) RNA made in vitro from an SP6 plasmid as follows. The CsCl pellet RNAs from the cytoplasmic and nuclear fractions were suspended in 100 μl of ET (1 mM EDTA–10 mM Tris hydrochloride [pH 7.4]) and treated by one of the following procedures: (i) Samples (9 μl) were self-annealed in a total volume of 20 μl of 2.5 × buffer A (0.375 M NaCl, 25 mM Tris chloride [pH 7.4], 2.5 mM EDTA) for 3 h at 65°C and diluted to 100 μl of the same buffer, and the total radioactivity was determined by trichloroacetic acid precipitation. (ii) Samples (9 μl) were treated as in (i) but diluted to 100 μl of 2.5× buffer A containing 8 μg of RNase A (a ratio of RNase to RNA of 1:5) and incubated for 20 min at 25°C before trichloroacetic acid precipitation, to determine the background resistance. (iii and iv) Samples (9 μl) were annealed in the presence of an estimated 1.5× and 2.5× excess, respectively, of LaC (−) RNA (prepared in vitro with an SP6 plasmid containing nucleotides 196 to 765 of the LaC S genome, i.e., the internal *Pst*I fragment). The excess of cold riboprobe to LaC S mRNA was estimated on the assumption that mRNA represented 5% of the total cytoplasmic CsCl pellet RNA and that LaC S mRNA represented 1% of the mRNA. The results of such an experiment with RNA from cells which were labeled for 30 min at 5 h p.i. shows that 87% of the S (+) RNA protected by the probe was found in the cytoplasmic fraction, whereas only 12% was found in the nuclear fraction (Table 1). More importantly, this distribution of pulse-labeled RNA did not change even when the pulse was lowered to as short a time as 3 min (Table 1). The proportion of radioactive LaC S (+) RNA in each cellular fraction as a function of pulse time is also shown in Table 1. Note that there is no indication of any displacement of the newly synthesized RNA between cellular compartments with time. These results are in sharp contrast to those of Herz et al. (5), who, in an analogous experiment with influenza virus-infected BHK cells which were fractionated similarly, found that 90% of the newly synthesized influenza virus mRNA in a 2-min pulse was located in the nuclear fraction and that 60% was still in the nuclear fraction after a 6-min pulse.

An average of 30.8% of the four pulse-labeled cytoplasmic RNAs were protected by an excess of the (−) S RNA probe, whereas an average of only 4.2% of the nuclear RNAs were protected (Table 1). It should be noted, however, that under the conditions of the experiment only about half of the mRNAs made are S genome specific (data not shown), and that the LaC S (−) RNA probe used contained only 570 nucleotides complementary to the S mRNA, which has been estimated to be 886 nucleotides long (10). When the above numbers are corrected accordingly, we estimate that an average of 97% of the pulse-labeled cytoplasmic RNAs were LaC specific, whereas an average of only 13% of the nuclear RNAs were virus specific. We have also controlled the relative purity of our cellular fractions in parallel experiments in which LaC-infected cells were labeled for longer times in the absence of Act D and the RNAs from each

TABLE 1. Cellular location of pulse-labeled LaC S mRNA[a]

Fraction	Pulse time (min)	cpm (%)				% of cpm in fraction
		Total	1.5 × probe	2.5 × probe	Avg protected	
Cytoplasm	3	4,768 (100)	1,097	1,168	1,133 (23.8)	85.3
	6	3,492 (100)	1,398	1,406	1,402 (40.1)	84.2
	15	29,495 (100)	9,045	8,655	8,851 (30.0)	87.5
	30	11,787 (100)	3,507	3,474	3,489 (29.6)	87.8
Nucleus	3	7,017 (100)	208	182	195 (2.8)	14.7
	6	4,153 (100)	328	198	263 (6.3)	15.8
	15	46,352 (100)	1,397	1,122	1,260 (2.7)	12.5
	30	9,603 (100)	765	204	486 (5.1)	12.2

[a] All determinations were carried out in duplicate. The background resistance in the absence of added probe was found to be between 5 to 10% of the total counts per minute for both the cytoplasmic and nuclear RNAs and has been subtracted from the numbers shown for each pulse-labeled experiment.

cellular fraction were examined by sucrose gradient centrifugation. The cytoplasmic fraction contained no detectable RNA greater than 28S, and the nuclear fraction did contain radioactivity in the 18S region of the gradient, although this did not represent a distinct peak (Fig. 2). Summing of the 18S radioactivity in the gradient (fractions 20 to 22, horizontal bar above) showed that 19,800 cpm out of the total of 132,000 cpm of the total cellular RNA which sediments at 18S was found in the nuclear fraction. Using the presence of 45S rRNA in the cytoplasmic fraction as a guide to nuclear contamination and the presence of 18S rRNA in the nuclear fraction as a sign of cytoplasmic contamination, we estimate that our cytoplasmic fractions were essentially pure, whereas our nuclear fractions were contaminated with 10 to 15% cytoplasm. The small amount of pulse-labeled LaC RNA which we find in the nuclear fraction can thus be explained entirely by cytoplasmic contamination.

Two further points should be taken into consideration in analyzing the data. (i) As pointed out by Herz et al. (5), the large 45S rRNA is not necessarily a good marker for nuclear leakage of other RNAs which are much smaller. Our fractionation experiment detailed in Fig. 2 demonstrates that few if any of the nuclei were ruptured during the fractionation procedure, but it does not take into account the possibility that the nuclear membranes contained holes large enough for the LaC S mRNA to escape completely but not large enough to allow any of the 45S rRNA to leak out. Such hypothetical holes in the nuclear membrane would not seem to be due to the fractionation procedure itself, since Herz et al. (5), using the same procedure on the same cells infected with influenza virus, found that the majority of the small influenza virus mRNAs labeled in a short pulse were contained in the nuclear fraction. We cannot however rule out the possibility that such holes in the nuclear membrane are the result of the LaC infection. In this case, however, even the repetition of the influenza virus localization experiment would not serve as an adequate control for our LaC-infected BHK cells. (ii) As mentioned above, the cytoplasmics RNAs were isolated

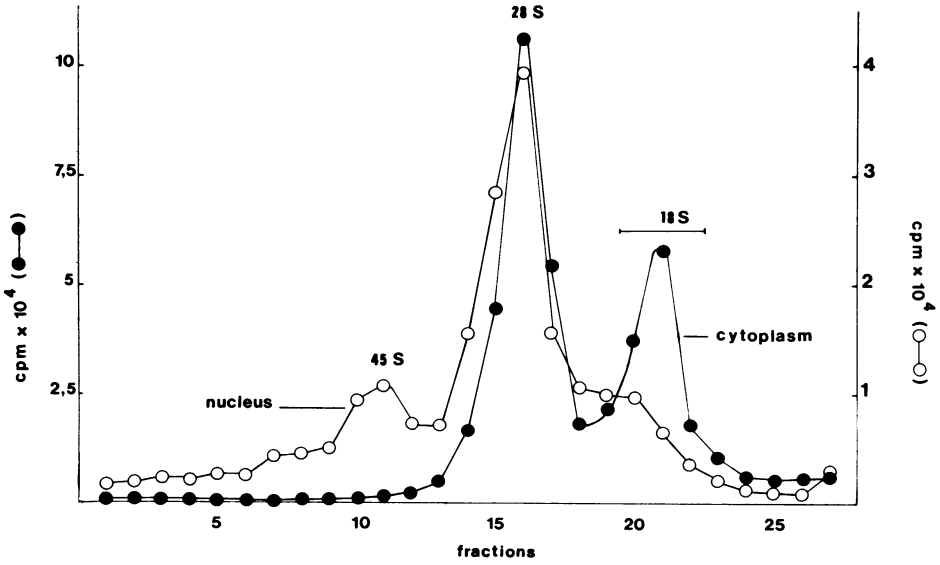

FIG. 2. Control of the cellular fractionation procedure. Similar cultures of LaC-infected BHK cells as detailed for Table 1 were labeled with 50 μCi of [^{3}H]uridine per dish from 1 to 5 h p.i., except that the cultures were not treated with Act D or glucoseamine. The cells were harvested and fractionated as described for Table 1, and 10% of the yield from one dish of both the cytoplasmic and nuclear fractions was centrifuged on 5 to 23% sucrose velocity gradients.

under conditions where the genome and antigenome RNAs do not pellet through the CsCl density gradient, but the nuclear RNAs contained all of the LaC RNAs. Further, under our experimental conditions, the steady-state, cytoplasmic, encapsidated LaC RNAs which band in CsCl density gradients are composed of approximately 30% antigenomes and 70% genomes. For the determination of percent S genomes and antigenomes in LaC-infected BHK cells, banded nucleocapsids labeled with [^{3}H]uridine were separated into L, M, and S RNAs by centrifugation, and self-annealing of the S RNA was determined by standard methods. In duplicate determinations, the results were as follows: no RNase, 4,229 cpm (100%); with no self-annealing, 117 cpm (2.8%); with 4 h of self-annealing, 2,515 cpm (59.4%); with 5 h of self-annealing, 2,546 cpm (60.2%). Similar results were also obtained for the L and M nucleocapsid RNAs (data not shown) (10). The majority species of the S nucleocapsid RNAs was shown to be (−) genomes, since self-annealed S RNA would still anneal to (+) but not to (−) S genome riboprobes (data not shown).

Should these encapsidated RNA species also exist in the nucleus, the preexisting pool of excess genomes could anneal to the pulse-labeled mRNA during isolation, and these mRNAs would then not be available for annealing with the probe. If this were so, however, and if nuclear LaC S (+) RNA represented a significant fraction of the newly synthesized RNA, it would still be visible, but as an increase in the RNase-resistant radioactivity recovered in the absence of added probe. This RNase-resistant fraction (which has been subtracted as a baseline in all the calculations shown in Table 1), however, routinely represented only 5 to 10% of the total radioactivity and was the same in RNA from either cellular compartment. Within the limits of the cellular fractionation noted above, the results of our experiments would thus indicate that LaC S mRNA is made entirely in the cytoplasm, consistent with the finding that the LaC endonuclease uses a stable pool of cellular mRNAs as a source of capped primers.

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LITERATURE CITED

1. **Bishop, D. H. L., C. H. Calisher, J. Casals, M. T. Chumakov, S. Y. A. Gaidamovich, C. Hannoun, D. K. Lvov, I. D. Marshall, N. Oker-Blom, R. F. Pettersson, J. S. Porterfield, P. K. Russell, R. E. Shope, and E. G. Westaway.** 1980. Bunyaviridae. Intervirology **14**:125–143.
2. **Bishop, D. H. L., M. E. Gay, and Y. Matsuoko.** 1983. Nonviral heterogenous sequences are present at the 5′ ends of one species of snowshoe hare bunyavirus S complementary RNA. Nucleic Acids Res. **11**:6409–6418.
3. **Chirgwin, J. M., A. E. Przbyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**: 5294–5299.
4. **Goldman, N., I. Presser, and T. Sreevalson.** 1977. California Encephalitis virus: some biological and biochemical properties. Virology **76**:352–364.
5. **Herz, C., E. Stavnezer, R. M. Krug, and T. Gurney.** 1981. Influenza virus, an RNA virus, synthesizes its messenger RNA in the nucleus of infected cells. Cell **26**:391–400.
6. **Lamb, R. A., and P. W. Choppin.** 1983. The gene structure and replication of influenza virus. Annu. Rev. Biochem. **52**:467–506.
7. **Krug, R. M.** 1981. Priming of influenza viral RNA transcription by capped heterologous RNAs. Curr. Top. Microbiol. Immunol. **93**:125–149.
8. **Obijeski, J. F., and F. A. Murphy.** 1977. Bunyaviridae: recent biochemical developments. J. Gen. Virol. **37**:1–14.
9. **Patterson, J. L., B. Holloway, and D. Kolakofsky.** 1984. La Crosse virions contain a primer-stimulated RNA polymerase and a methylated cap-dependent endocnuclease. J. Virol. **52**:215–222.
10. **Patterson, J. L., and D. Kolakofsky.** 1984. Characterization of La Crosse virus small-genome transcripts. J. Virol. **49**:680–685.
11. **Pennington, T. H., and C. R. Pringle.** 1978. Negative strand viruses in enucleate cells, p. 457–464. *In* B. W. J. Mahy and R. D. Barry (ed.), Negative strand viruses and the host cell. Academic Press, Inc., New York.