JOURNAL OF VIROLOGY, Jan. 1975, p. 36-40

Vol. 15, No. 1
Printed in U.S.A.

RNA Synthesis in Cells Infected with Herpes Simplex Virus

IX. Evidence for Accumulation of Abundant Symmetric Transcripts in Nuclei

M. KOZAK AND B. ROIZMAN*

Departments of Microbiology and Biophysics, The University of Chicago, Chicago, Illinois 60637

Received for publication 3 September 1974

RNA extracted from nuclei of 8-h infected cells drove approximately 50% of herpes virus DNA into DNA-RNA hybrid. The same RNA, preannealed under conditions which allowed base pairing to take place, drove only 35% of the DNA into DNA-RNA hybrid; further annealing of the RNA did not diminish the amount of RNA sequences remaining available for subsequent hybridization with DNA. Upon denaturation of the preannealed RNA, the RNA sequences sequestered during preannealing became available again for hybridization with DNA. The base pairing that occurred during preincubation of the RNA was intermolecular, since it was RNA concentration dependent and was not affected by limited alkaline hydrolysis. The nuclear viral transcripts that remained available for hybridization, after preannealing of the RNA, were a subset of the RNA sequences that accumulated in the cytoplasm of infected cells. In addition, a small amount (derived from 5% or less of the viral DNA) of complementary transcripts was detected in the cytoplasm.

During productive infection of HEp-2 cells by human herpes virus I (herpes simplex virus 1, HSV-1), RNA complementary to about 50% of the viral DNA was readily detected in the nuclei, whereas only 40 to 42% of the DNA sequences were represented in cytoplasmic transcripts (M. Kozak and B. Roizman, Proc. Nat. Acad. Sci. U.S.A., in press). The experiments described in this paper revealed a further qualitative difference between nuclear and cytoplasmic viral transcripts, namely, the presence of complementary RNA sequences in infected cell nuclei, detected by reversible loss of ability to drive a portion of labeled viral DNA into DNA-RNA hybrid when RNA was allowed to self-anneal prior to hybridization with DNA. In contrast, complementary RNA sequences appeared to be present in only trace amounts in the cytoplasm of infected cells.

MATERIALS AND METHODS

Cells and virus. The F strain of HSV-1 was passaged at low multiplicity a maximum of four times in HEp-2 cells. Procedures for cell maintenance and infection have been described previously (9).

Viral DNA. The procedures for purifying viral DNA and for labeling it in vitro with [^{3}H]TTP by repair synthesis, using *Escherichia coli* DNA polymerase I, were the same as those described previously (Kozak and Roizman, in press). The DNA preparations used in these studies had specific activities of 1 $\times 10^6$ to 2 $\times 10^6$ counts per min per μg. Comparison of in vitro and in vivo labeled viral DNA (Kozak and Roizman, in press) revealed no difference in hybridization with viral RNA, indicating that the DNA was randomly labeled in vitro.

Extraction of RNA from infected cells. Confluent monolayers of HEp-2 cells in roller bottles were infected with 10 PFU of virus per cell, washed with phosphate buffered saline, and resuspended in buffer A which contained 10 mM Tris-hydrochloride (pH 7.5), 50 mM KCl, 2.5 mM $MgCl_2$, and 0.2 mM EDTA. The cells were lysed by addition of an equal volume of 2% Nonidet P-40 in buffer A followed by gentle swirling for 15 min at 4 C. After pelleting the nuclei at low speed, the cytoplasm was recentrifuged to remove debris, and then dialyzed at 4 C against 0.1 M Tris-hydrochloride (pH 9) in preparation for phenol extraction.

Nuclei were washed twice at 4 C with 1% Nonidet P-40, resuspended in 5 mM Tris-hydrochloride (pH 6.9), 5 mM $MgCl_2$, and lysed by addition of sodium deoxychlolate to a final concentration of 1.5 to 2%. The lysate was treated with pancreatic DNase (RNase-free DNase I from Worthington, 50 μg/ml) for 45 min at 37 C. After adjusting the pH to 9.0 with 0.15 M Tris-hydrochloride, RNA was extracted with phenol and 0.5% sodium dodecyl sulfate at 56 C, followed by extraction with 2% isoamyl alcohol in chloroform at room temperature. RNA was precipitated from ethanol, and then dialyzed at 4 C against 5 mM Tris-hydrochloride (pH 6.9). The entire cycle of DNase treatment, phenol and chloroform extractions, and ethanol precipitation was repeated two more times. The final RNA preparation was then dialyzed

against 0.04 M sodium phosphate (pH 6.8). Reconstruction experiments with labeled viral DNA and excess cellular RNA established that, under the conditions used, pancreatic DNase hydrolyzed all traces of viral DNA.

In one experiment, a sample of the RNA was subjected to limited alkaline hydrolysis prior to the final phenol extraction. The RNA was held for 1 h at 4 C in 0.16 N NaOH, 0.1 M NaCl, 0.01 M EDTA, and 0.01 M Tris, and then neutralized by addition of 0.5 M NaH_2PO_4.

DNA-RNA hybridization. Hybridization was done in liquid with trace amounts of labeled viral DNA and excess unlabeled RNA, such that the hybridization reaction was driven by RNA (4). Experiments with preannealed RNA were done as follows. A solution of RNA containing 0.32 M Na^+ was incubated at 75 C in sealed glass capillary pipettes for 4 to 12 h. The RNA was then added to heat-denatured ^{3}H-labeled viral DNA and hybridized for 2 to 3 h at 75 C in 0.23 M NaCl and 0.04 M sodium phosphate buffer (pH 6.8). The total Na^+ concentration in the hybridization reaction mixtures was 0.29 M. Viral RNA concentrations ranged from 8 to 25 mg/ml during preannealing, and from 0.02 to 10.7 mg/ml during hybridization with DNA. The total concentration of RNA in each hybridization mixture was adjusted by addition of uninfected HEp-2 RNA, so that each mixture contained the same final RNA concentration. After hybridization, the fraction of DNA driven into DNA-RNA hybrid was assayed by digesting with the single strand-specific *Neurospora crassa* nuclease (7), and then precipitating the enzyme-resistant ^{3}H-labeled DNA with trichloroacetic acid. The precipitate was collected on a membrane filter (Millipore Corp.) and counted. DNA reassociation during the course of the DNA-RNA hybridizations was monitored by incubating viral DNA in the presence of uninfected HEp-2 RNA; DNA reassociation did not exceed 2% during the 2- to 3-h duration of these experiments.

RESULTS

Demonstration of self-annealing viral RNA sequences in nuclei of infected cells. RNA extracted from the nuclei of 8-h infected cells hybridized with about 50% of the viral DNA sequences (Fig. 1). The same RNA, after preincubation under conditions which allowed the formation of RNA-RNA hybrids, drove only about 35% of the DNA into DNA-RNA hybrid

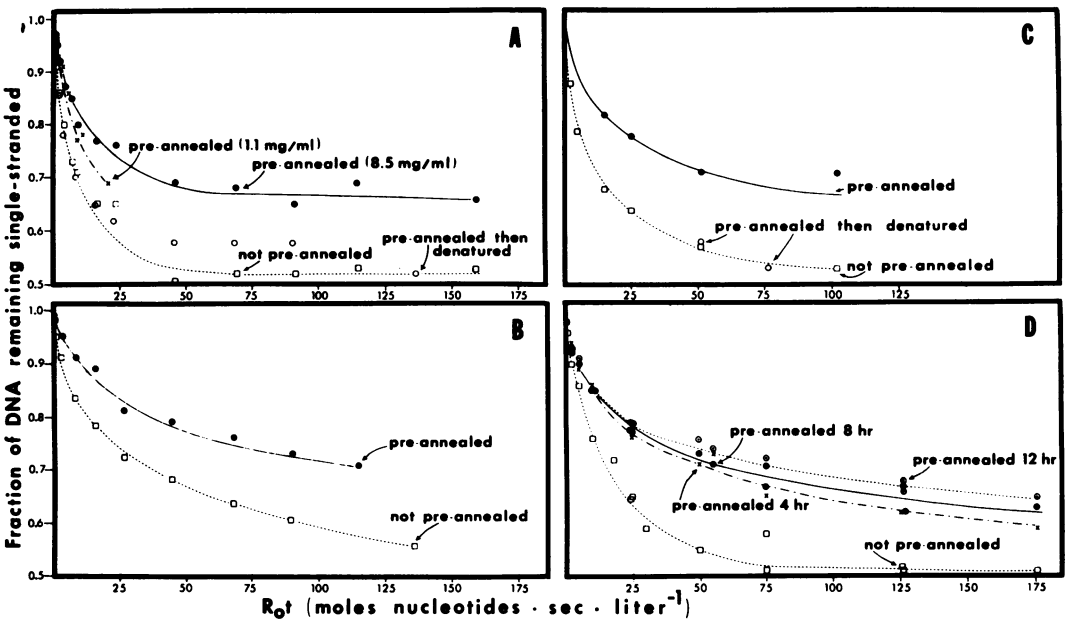

FIG. 1. *Reversible loss of hybridizable sequences upon preannealing viral RNA. RNA extracted from nuclei of 8-h infected cells was hybridized with ^{3}H-labeled viral DNA. A portion of the RNA was hybridized without pretreatment (□), another portion of RNA was self-annealed at 75 C before hybridization with DNA (●, ×, ⊙), and a third portion was preannealed and then denatured at 107 or 115 C before hybridization with DNA (○). Unless otherwise stated, the concentration of Na^+ was 0.32 M during preannealing and 0.29 M during hybridization with DNA. (A) RNA was preannealed for 10 h at a concentration of 8.5 mg/ml (●) or 1.1 mg/ml (×). The RNA preparations were diluted, and each point was hybridized for 2 h to give the indicated R_0t. (B) RNA (8.5 mg/ml) was preannealed for 10 h in 0.106 M Na^+ and then hybridized with DNA for 2 h in 0.1 M Na^+. (C) RNA that had been fragmented by limited alkaline hydrolysis was preannealed at a concentration of 8 mg/ml for 12 h; the duration of the subsequent hybridization with DNA was 2.4 h for each point. (D) The RNA concentration was 19 mg/ml during the preincubation, which was carried out for 4 (×), 8 (●), or 12 h (⊙). The duration of hybridization with DNA was 2 h for each point.*

(Fig. 1A). However, brief exposure of the self-annealed RNA to denaturing temperatures prior to hybridization with DNA restored its ability to drive 50% of the DNA into DNA-RNA hybrid, indicating that the sequences sequestered during self-annealing of the RNA again became available for hybridization with viral DNA. In experiments with six different nuclear RNA preparations, 76 to 100% of the sequestered sequences were restored by denaturing the RNA prior to hybridization with DNA (Fig. 1A and C, Table 1). The reversible loss of hybridizable sequences upon incubation of RNA under annealing conditions indicated the presence in the nuclei of infected cells of complementary viral RNA sequences that existed in single-stranded form prior to in vitro annealing.

Nature of the self-annealing viral RNA sequences. Three types of experiments bear on the question of whether the base pairing occurred between complementary regions within a single RNA molecule which folded back upon itself to form a hairpin-like structure or whether the complementary sequences resided in separate RNA chains. (i) As shown in Fig. 1B, RNA preincubated in 0.106 M Na^+ at 75 C, which is 18 C below the T_m of HSV-1 DNA in this salt, underwent the same loss of hybridizable sequences as RNA preincubated in 0.32 M Na^+ at 75 C, 25 C below the T_m. Self-annealing of viral RNA sequences thus occurred under conditions which should have minimized formation of duplexes by poorly matched base pairs. (ii) As the RNA concentration during preincubation was increased from 1.1 to 8.5 mg/ml, more RNA was sequestered and became unavailable for subsequent hybridization with viral DNA (Fig. 1A). Thus, self-annealing of the viral RNA was concentration dependent. (iii) As shown in Fig. 1C, fragmentation of the RNA by limited alkaline hydrolysis did not reduce the reversible loss, during preincubation, of RNA sequences available for subsequent hybridization with viral DNA. The conditions employed for alkaline hydrolysis (described in Materials and Methods), would be expected to reduce the size of the RNA to fragments approximately 200 nucleotide residues long (5). This should have converted intrastrand folding from a unimolecular to a bimolecular reaction, thereby greatly reducing its rate. The failure of limited fragmentation to reduce or abolish self-annealing of the RNA is consistent with the hypothesis that annealing occurred between complementary sequences in separate RNA chains, rather than by intrastrand folding.

TABLE 1. *Restoration of ability to drive labeled DNA into DNA-RNA hybrid, after denaturation of preannealed RNA*

RNA prepn	$R_0 t$[d]	Viral DNA (%) driven into hybrid by:		
		Untreated RNA	Preannealed RNA[a]	Preannealed and denatured RNA
1[b]	58	46	31	44
	106	52	37	50
2[b]	80	44	33	40
	107	53	32	48
3[b]	25	35	23	30
	75	42	28	41
	126	48	33	43
4[c]	91	51	43	50

[a] Preannealing was carried out at 75 C in 0.32 M Na^+ for 10 h.

[b] RNA extracted from nuclei of 8-h infected cells. The RNA concentration during preannealing was 7 to 20 mg/ml.

[c] RNA extracted from nuclei of 14-h infected cells. The RNA concentration during preannealing was only 4.9 mg/ml; hence self-annealing of complementary RNA sequences probably did not reach completion.

[d] RNA concentration (moles of nucleotide per liter) × time (in seconds) (reference 4).

The RNA sequences sequestered during preannealing arise from a limited portion, approximately 15%, of the viral DNA. This is suggested by the observation that the hybridization reaction between DNA and RNA that had been preincubated leveled off after about 35% of the DNA was driven into hybrid (Fig. 1A), and by the observation that the concentration of the residual sequences that remained available for hybridization with DNA was not significantly reduced even when the preincubation was prolonged to 8 or 12 h at an extremely high RNA concentration (Fig. 1D).

Search for complementary sequences in cytoplasmic RNA. RNA sequences contained in the cytoplasm of 8-h infected cells hybridize with a maximum of 42% of the viral DNA (Kozak and Roizman, in press). Two experiments were designed to determine whether complementary viral RNA sequences were present in the cytoplasm of infected cells. The first experiment was a summation hybridization between cytoplasmic RNA and nuclear RNA that had been preincubated for 8 h to eliminate the self-annealing sequences. Table 2 shows that the amount of viral DNA driven into hybrid by both cytoplasmic and preannealed nuclear RNA did not significantly exceed the amount of hybrid formed when each RNA was reacted

individually. Thus, all the nuclear RNA sequences that remained available for hybridization after preannealing were also present in the cytoplasm of infected cells. The sequences that selectively accumulated in the nuclei and failed to reach or accumulate in the cytoplasm were, therefore, derived from the self-annealing fraction of intranuclear viral transcripts.

Figure 2 shows that RNA sequences reactive with about 5% of the viral DNA were reversibly lost during preincubation of the cytoplasmic RNA. It appears, therefore, that in addition to the non-self-annealing nuclear sequences derived from 35% of the viral DNA, a small amount of complementary transcripts, corresponding to about 5% of the DNA sequences, also entered the cytoplasm.

TABLE 2. *Summation hybridization between cytoplasmic and preannealed nuclear RNA*[a]

Cytoplasmic RNA	Preannealed nuclear RNA[b]	Viral DNA (%) in hybrid		
		Observed	Predicted for summation of:	
			Identical sequences	Non-identical sequences
192[c]		38		
339		41		
	111[c]	35		
192	111	42	41	42-50
192	111	40	41	42-50

[a] Denatured, ^{3}H-labeled viral DNA was hybridized for 3 h with one or both RNA preparations, extracted from 8-h infected cells.

[b] The nuclear RNA, preannealed for 8 h, was a sample of the same preparation used in Fig. 1D. Higher concentrations of this RNA resulted in no further hybridization.

[c] Numbers indicate R_0t.

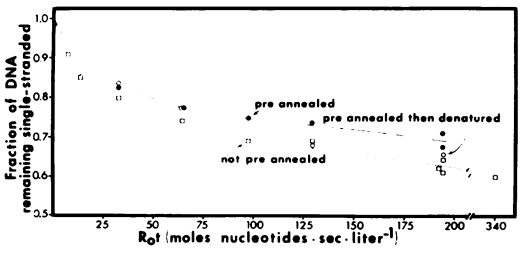

FIG. 2. *Effect of preannealing on ability of cytoplasmic RNA to hybridize with viral DNA. A portion of the RNA was hybridized without pretreatment* (□). *The remainder was preincubated for 8 h at a concentration of 24.5 mg/ml* (●). *A portion of the preannealed RNA was denatured at 115 C prior to hybridization* (○). *The duration of hybridization with DNA was 2 h for all points.*

DISCUSSION

We have reported in this paper the observation that nuclei of herpes virus-infected cells contain high concentrations of complementary RNA transcripts arising from a limited portion of the DNA, and that cytoplasmic RNA contains only trace amounts of such sequences.

There are two possible explanations for the origion of the self-annealing RNA. One postulates that complementary transcripts arise from 15% of the DNA, i.e., sequences derived from 7.5% of each DNA strand accumulate at nearly equimolar concentrations in the infected cell nuclei. The alternative is that complementary RNA sequences are derived from 15% of each DNA strand, but the transcripts arising from one strand are present at a lower concentration than the corresponding RNA sequences derived from the opposite strand. Preannealing would sequester the less abundant RNA in an RNA-RNA hybrid, but the complementary sequences would remain available for hybridization with DNA, although their concentration would have been reduced. We cannot at present differentiate between these two hypotheses.

The experiments presented in this paper revealed RNA, capable of self-annealing, that was derived from a limited portion of the viral DNA. The hybridization technique employed in these studies, however, allowed detection of only those species of RNA which were present in vast excess over the DNA probe. We cannot eliminate the possibility that the entire viral genome might be transcribed symmetrically. Given a sufficient disparity in concentration of the complementary sequences, the small reduction in concentration of the excess strand that would have occurred during preannealing would not have been detectable. In fact, preliminary studies involving purification of RNase-resistant viral RNA suggest that a large percentage of the viral DNA gives rise to such RNA, and hence might be symmetrically transcribed (B. Jacquemont and B. Roizman, unpublished observations).

The absence of significant amounts of complementary RNA from the cytoplasm of infected cells reinforces the earlier observation that only a subset of the nuclear viral transcripts are present in the cytoplasm (Kozak and Roizman, in press). Thus, there is discrimination among viral transcripts, occurring either at the level of transport from nuclei to cytoplasm or at the level of RNA stability in the cytoplasm.

The presence of complementary RNA sequences in infected and uninfected eukaryotic

cells has been well documented and offers no surprise. Complementary transcripts arising from symmetrical transcription of simian virus 40 (1) and vaccinia virus (3) have been reported. In uninfected eukaryotic cells, the complementary sequences exist in the form of hairpin loops due to intramolecular folding (5). This is in contrast with the situation observed with vaccinia virus (3) and with the present observations on herpes virus, in which complementary viral sequences appear to reside in separate RNA molecules. It is noteworthy that retention of the bulk of the self-annealing sequences to the nuclei, observed here with herpes virus-infected cells, is similar to the intracellular distribution of double-stranded RNA reported for uninfected cells (2, 6). The presence in the cytoplasm of eukaryotic cells of a nuclease that preferentially degrades double-stranded RNA could be significant in this regard (8).

ACKNOWLEDGMENTS

These studies were done under the auspices of the Chicago Cancer Research Center Project (CA-14599), and were aided by grants from the American Cancer Society (VC 103 H), the Public Health Service National Cancer Institute (CA 08494), and the National Science Foundation (GB 38799), and by an appropriation from the American Cancer Society Institutional Grant IN-41-N. M. K. is a fellow of the Damon Runyon-Walter Winchell Cancer Fund.

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