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Frog Virus 3 Requires RNA Polymerase II for Its Replication

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The involvement of host cell RNA polymerase II in the replication of frog virus 3 (FV 3) was examined in α-amanitin-sensitive or -resistant Chinese hamster ovary (CHO) cells in the presence and absence of α-amanitin. In the presence of α-amanitin, FV 3 replicated normally in resistant CHO cells but failed to do so in sensitive CHO cells. Synthesis of virus-specific RNAs and proteins was inhibited in sensitive cells infected in the presence of α-amanitin, but in α-amanitin-resistant cells, as expected, virus-specific protein synthesis and, by implication, virus-specific RNA synthesis were not affected by the presence of the drug. Inhibition of FV 3 replication was maximum when α-amanitin was added to sensitive CHO cells before virus adsorption, but the drug had no effect on virus replication if added after the adsorption. These data indicate that host RNA polymerase II was required for early transcription of the FV 3 genome and confirm a nuclear requirement for FV 3 RNA synthesis (R. Goorha et al., Virology 82:34-52, 1978).

Frog virus 3 (FV 3) is a large, icosahedral, cytoplasmic DNA virus belonging to the family Iridoviridae (12). Members of this family have been thought to replicate exclusively in the cytoplasm (10). If true, this would imply that FV 3 virions contain an RNA polymerase that would synthesize viral RNA in the cytoplasm. Therefore, a great deal of effort has been directed towards discovery of an RNA polymerase in the virion, but the results have been equivocal. Gaby and Kucera (5) reported a low level of transcriptase activity in the virus particles, but this activity could be detected only if "activated" calf thymus DNA was present in the reaction mixture. In the absence of this exogenous template, there was no polymerase activity. Other research workers have failed to find any RNA polymerase activity in the virions (D. Willis, personal communication; A. Aubertin, personal communication; Furuichi and Goorha, manuscript in preparation). Moreover, over the past several years we have obtained data which question the cytoplasm as the sole site of RNA synthesis (8, 9). The evidence can be summarized as follows: (i) FV 3 did not grow in enucleated cells, nor could any virus-specific RNA or protein synthesis be detected in cells that had been enucleated before infection (9); (ii) electron microscopic autoradiographic data show that at least 85% of the newly synthesized viral RNA is in the nucleus (8); (iii) early viral RNAs display internal methylation of adenosine which is characteristic of cellular or viral mRNA's synthesized in the nucleus (13a). These data suggest that early FV 3 transcription occurs in the nucleus. However, the source of enzyme (viral or cellular) for early transcription in FV 3-infected cells has not been established.

Recently, mutant cell lines that possess α-amanitin-resistant RNA polymerase II have become available (1, 4, 15). These cells provided an opportunity to determine whether viral or cellular RNA polymerase II is used for FV 3 transcription. If, in the presence of α-amanitin, FV 3 replicates in mutant cells but not in sensitive cells, it would indicate that the virus utilizes all or part of the cellular RNA polymerase II for its transcription. If the virus possesses its own transcriptase, the effect of α-amanitin would be identical in both the sensitive and resistant cells. I have used a mutant Chinese hamster ovary (CHO) cell line which not only has α-amanitin-resistant RNA polymerase II (4), but also supports replication of FV 3.

Mutant CHO cells resistant to α-amanitin (CHO-AmaI) and parental cells sensitive to the drug [CHO(S)] were grown as monolayers in α medium (GIBCO) with 10% fetal calf serum supplemented with nonessential amino acids in 60-mm dishes. Cells were infected with FV 3 (3 PFU/cell) and incubated at 30°C. Virus replication occurred in both cell types (final titer, 50 to 100 PFU/cell), and newly produced virus was quantitated by plaque assay in fathead minnow cells (13). Maximum yields were obtained by 18 h.

The effects of various concentrations of α-amanitin, when added before adsorption, on the growth of FV 3 in CHO cells are shown in Fig. 1. About 90% inhibition of virus growth occurred at a concentration of 20 μg of α-amanitin per ml. The same concentration of the drug had no

significant effect on FV 3 replication in CHO-AmaI cells. Initially, there was considerable variation in the extent of inhibition of FV 3 replication from one experiment to another. However, reproducible results were obtained if the multiplicity of infection was kept low (2 to 3 PFU/cell) and virus replication was not allowed to proceed for more than one replication cycle (data not shown). The results demonstrate that cellular RNA polymerase II activity is essential for FV 3 replication.

I next investigated the time during the infectious cycle when FV 3 replication was most susceptible to the action of α-amanitin. Figure 2 shows that the maximum inhibition of virus

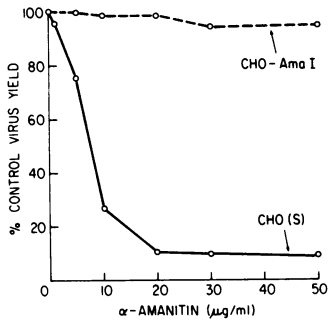

FIG. 1. *Inhibition of FV 3 replication by α-amanitin. Replicate CHO(S) or CHO-AmaI cell cultures were exposed to FV 3 (3 PFU/cell) for 60 min at room temperature. The cells were washed three times after the removal of unadsorbed virus, supplemented with fresh medium containing various concentrations of α-amanitin, and incubated at 30°C. Cells which received α-amanitin were exposed to the drug 60 min before the adsorption of virus, and α-amanitin was also present during the adsorption period. After 18 h of incubation at 30°C, virus yields were determined by plaque assay. The results are expressed as percentage of virus yield of untreated cultures.*

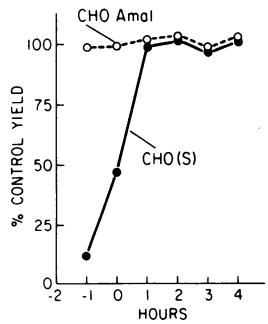

FIG. 2. *Effect of time of addition of α-amanitin on FV 3 replication. Cells were infected as described in Fig. 1. At indicated times before or after infection, the medium was supplemented with α-amanitin (20 μg/ml). Other details as described in Fig. 1.*

replication occurred when cells were treated with the drug for at least 60 min before infection with FV 3. If the cells were exposed to virus and α-amanitin simultaneously, inhibition of virus replication was considerably reduced, but no inhibition at all occurred if the drug was added at any time after virus adsorption. These results suggest that RNA polymerase II is required only very early in the infectious cycle of FV 3.

The most obvious requirement of FV 3 for the host RNA polymerase in replication would be the use of RNA polymerase II for FV 3 transcription. The time course of inhibition (Fig. 2) suggests that the enzyme is required during the early transcriptional phase of virus replication. I therefore examined FV 3 transcription in CHO(S) cells in the presence or absence of α-amanitin. It had been previously shown that FV 3 severely inhibits cellular RNA and protein synthesis (6, 16) and that virus-specific RNAs and proteins can be resolved into 47 and 35 bands, respectively, by polyacrylamide gel analysis under denaturing conditions (16). As seen in Fig. 3, virus-specific RNA synthesis could be detected at a low level 1 h postinfection in FV 3-infected CHO(S) cells. By 3 or 7 h postinfection, viral RNAs were synthesized at high rates and 47 bands could be detected. However, in the presence of α-amanitin very little if any virus-specific RNA synthesis was observed. In the presence of the drug, there was an 85% reduction in total RNA synthesis when measured by acid-precipitable radioactivity (data not shown). These results show that RNA polymerase II activity is required for early FV 3 transcription.

The half-life of FV 3 mRNA is about 5 h (7), and each mRNA presumably participates in multiple rounds of translation before its eventual degradation. Thus, measurement of viral protein synthesis should be a very sensitive tool for the production of viral mRNA. Therefore, to substantiate further that RNA polymerase II activity is required for early FV 3 transcription, I examined FV 3-specific protein synthesis (Fig. 4). In sensitive cells without the drug, virus-specific protein synthesis was detected at both 2 and 8 h after infection. However, in the presence of α-amanitin very little virus-specific protein synthesis was detected. In resistant cells, as expected, the presence of α-amanitin had no effect on viral protein synthesis at either 2 or 8 h after the infection. These results confirm the previous conclusion that FV 3 transcription, at least in the early stage, is dependent upon RNA polymerase II activity.

The exact nature of this requirement for RNA polymerase II is not known. However, previous work on the effects of FV 3 on RNA polymerase

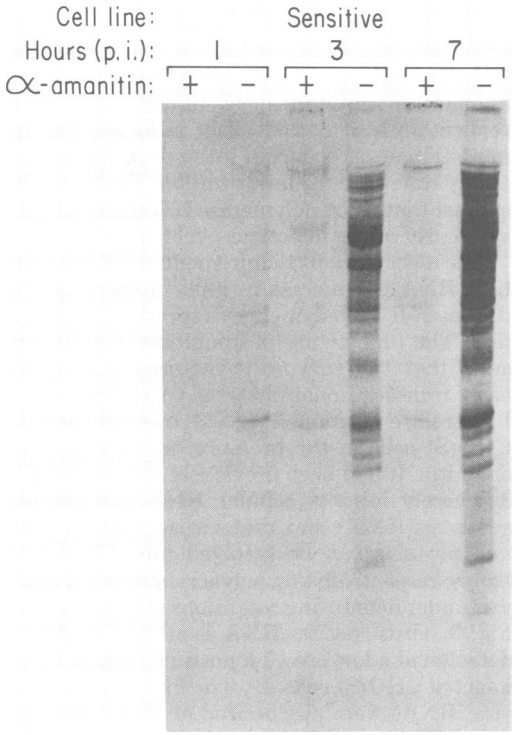

FIG. 3. *Inhibition of FV 3 transcription by α-amanitin. Cells were exposed to 50 μg of α-amanitin per ml for 1 h and then infected with 10 PFU/cell. RNA, labeled for 30 min with [^{3}H]uridine (50 μCi/ml; specific activity, 20 Ci/mmol) at indicated times, was extracted from the cytoplasm and subjected to electrophoresis on a 3.5% polyacrylamide gel under denaturing conditions (16).*

II provides some clues as to the probable function of this enzyme in FV 3 transcription. It has been reported that, in FV 3-infected cells, RNA polymerase II undergoes the following modifications: (i) there is a drastic reduction in the number of molecules able to bind α-amanitin; and (ii) the molecules still capable of binding α-amanitin display a lower affinity for the drug, although they retain a normal affinity for ribonucleotides (3). These modifications of the enzyme do not require virus replication; RNA polymerase II, purified from cells which had been exposed to viral proteins solubilized from FV 3 particles, also showed a reduced α-amanitin-binding capability. Therefore, it appears that a structural protein of FV 3 modifies polymerase II (2). Aubertin and co-workers (2) also suggested that the modifications by the viral protein inactivated the enzyme and that this inactivation was partially responsible for the inhibition of cellular RNA synthesis. I would like to extend this explanation and suggest that the modified RNA polymerase II not only has a reduced affinity for cellular DNA template, but now has a preference for the viral genome. Thus, if α-amanitin is present before infection with FV 3, then it binds to RNA polymerase II and prevents the subsequent viral modification of the enzyme; the result is inhibition of virus replication by α-

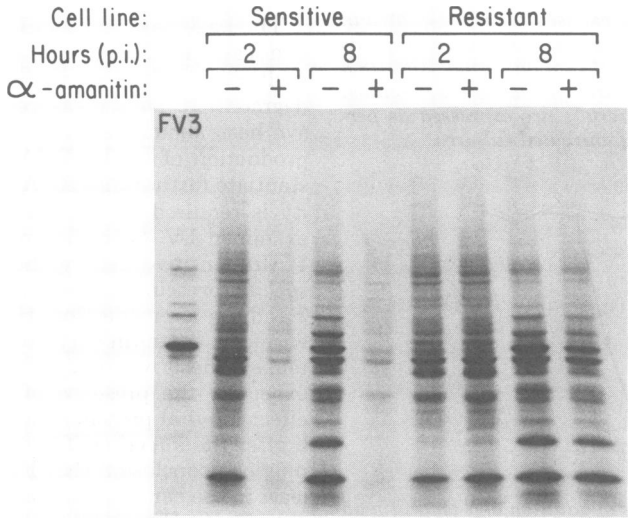

FIG. 4. *Inhibition of FV 3 protein synthesis by α-amanitin. Cells, exposed to α-amanitin, were infected with FV 3 as described in Fig. 3. At indicated times, proteins were labeled with [^{35}S]methionine (25 μCi/ml; specific activity, 600 Ci/mmol) for 30 min. Cytoplasmic extracts were prepared and electrophoresed on a 5 to 15% sodium dodecyl sulfate-gradient polyacrylamide gel as previously described (16).*

amanitin. However, if FV 3 is present before the addition of the drug, then a structural protein of FV 3 modifies RNA polymerase II for its own transcription and α-amanitin has no effect. Such a competition between α-amanitin and a structural protein of FV 3 would also explain why high multiplicities of the virus cause a partial reversal of α-amanitin action on FV 3 replication. However, it is unlikely that there is a direct competition between structural protein(s) of FV 3 and α-amanitin for a particular site on RNA polymerase II because the inhibition of RNA polymerase activity by solubilized proteins does not occur in vitro with solubilized RNA polymerases or nuclei (2). Under in vivo conditions, the structural protein of FV 3 probably modifies RNA polymerase II indirectly. Presumably, the modified RNA polymerase II is unable to bind α-amanitin.

The present data do not exclude the possibility that the inhibition of FV 3 replication by α-amanitin was due to the inhibition of synthesis of a cellular RNA species required for virus transcription. It has been suggested that the synthesis of a cellular RNA species by RNA polymerase II is required for primary transcription of the influenza virus genome (11). However, a requirement of cellular RNA synthesis for FV 3 transcription is unlikely because FV 3 transcription can be initiated when cellular RNA synthesis has already been reduced by 90% or more (16).

RNA polymerase II is localized in the nucleoplasm (14); thus, the utilization of this enzyme for FV 3 transcription corroborates our previous observation that the site of viral RNA synthesis, at least in the early stages of infection, is the nucleus (8).

In summary, the results demonstrate that RNA polymerase II is required for FV 3 transcription and thus for virus replication. The data are also consistent with the explanation that RNA polymerase II, modified by a structural protein of FV 3, is utilized for transcribing the viral genome.

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