SPECIFICITY OF ROUS SARCOMA VIRUS SYNTHESIS

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1. Introduction

Nucleoside analogues and inhibitors of nucleic acid synthesis have been successfully used to elucidate certain aspects of the infective cycle of Rous sarcoma virus**. The requirements of both DNA synthesis and DNA function in the establishment of infection and the production of progeny virions were deduced by the use of drugs which affected nucleic acid metabolism [1–5]. Recent studies with both bacteria and eukaryotic cells have demonstrated that certain nucleoside analogues can be utilized to demonstrate the diverse specifications of nucleotide polymerases [6, 7]. We were prompted by this earlier work to examine the effects on RSV RNA synthesis of a series of nucleoside analogues.

We report here a description of the effect of 5-fluorouridine on RSV production from transformed chick embryo fibroblasts. The results described show that at concentrations of FUR which depress cellular RNA synthesis by 90%, viral RNA synthesis and the production of virions continue at normal rates. Although the total amount of virus produced by FUR-treated cells is normal, the infectivity yield is decreased. There is also a significant decrease in the glycosylation of the viral glycoproteins.

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** *Abbreviations:*

FUR	:5-fluorouridine
FUMP	:5-fluorouridine-5′-phosphate
dFUMP	:5-fluoro-2′-deoxyuridine-5′-phosphate
TdR	:thymine-2′-deoxyriboside
CEF	:chick embryo fibroblast
RSV	:Rous sarcoma virus
SDS	:sodium dodecyl sulfate.
CEF-RSV	:chick embryo fibroblasts transformed by RSV.

2. Methods

2.1. *Cells and virus*

Chick embryo fibroblast cultures were prepared from 11–12 day embryos as described by Temin [8]. Secondary cultures were transformed at a high multiplicity with the Schmidt-Ruppin D strain of RSV described earlier [9]. The transformed cells had been infected for at least eight days before use. The techniques employed for the isolation and purification of RSV, infectivity titrations, and the analysis of effects of inhibitors on cell growth have been previously described [9]. Cell cultures were maintained in Eagle's minimal medium supplemented with 10% fetal calf serum unless otherwise specified.

2.2. *Macromolecule synthesis*

The incorporation of radioactive precursors into macromolecules was analyzed as described previously [10–12].

2.3. *Cell fractionation and analysis of cellular and nuclear RNA*

The method used for the preparation of nuclear and cytoplasmic fractions of transformed and normal chick fibroblasts was that of Brdar et al. [13]. RNA was extracted from the cytoplasmic and nuclear fractions according to the procedure of Perry and Kelley [14] modified by Brdar et al. [15]. The extracted nuclear and cytoplasmic RNA's were analyzed on acrylamide gel columns by electrophoresis [13, 15].

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Table 1
Macromolecule synthesis in the presence of FUR.

	[^{3}H]guanosine (cpm)		[^{3}H]valine (cpm)
	RNA	DNA	Protein
Control	280×10^3	18×10^3	11×10^3
FUR	25×10^3	1×10^3	4.8×10^3

Transformed cells (3×10^5) were plated in 60 mm petri dishes and allowed to attach and grow overnight. The next day FUR (10 μg/ml) and TdR (10 μg/ml) were added to the experimental cultures. After the pretreatment period of 24 hr, radioactive precursors were added to the experimental cultures; the control cultures received isotope at the time the experimental were given FUR and TdR. Protein synthesis was measured by incorporation of [^{3}H]valine (5 μCi/ml) into hot acid insoluble material; RNA and DNA synthesis by the incorporation of [^{3}H]guanosine (3 μCi/ml, 1 μg/ml).

2.4. *Analysis of viral proteins*

Viral proteins were analyzed by SDS-polyacrylamide gel electrophoresis according to the method of Compans et al. [16].

3. Results

The growth of both transformed and normal chick embryo fibroblasts is stopped by FUR at concentrations as low as 0.1 μg/ml. The effect is immediate and irreversible even after short times of incubation. Although the cells do not divide in the presence of the drug, they remain attached to the surface of the petri dish for at least four days after the initial exposure to FUR. The inhibitory action of FUR is unaffected by the addition of TdR to the culture medium. Therefore, growth inhibition is probably not due to the conversion of FUR to dFUMP.

Macromolecule synthesis slows progressively in cells exposed to FUR. After a 32 hr exposure (table 1) DNA synthesis is decreased by 95%, RNA synthesis by 91%, and protein synthesis by 57% compared with untreated control cultures. The relatively high rate of protein synthesis, over long periods of time, implies that mRNA is made at a correspondingly high rate.

The RNA's in both nuclei and cytoplasm formed after exposure of cells to FUR were examined by polyacrylamide gel electrophoresis. These results, which are presented elsewhere [13], show that there are no qualitative differences in the nuclear RNA species of control and FUR-treated cells. The synthesis of all nuclear RNA classes is decreased, but the synthesis of the heterogeneous RNA of high molecular weight (45 S) is decreased the least. In addition, there is little or no processing of the 45 S RNA to the lower molecular weight 32 S, 28 S or 18 S molecules [13].

The arrest in the processing of ribosomal RNA precursors in FUR-treated cells is most conspicuous on examination of the cytoplasmic RNA's. In comparison with controls, there is a decrease in newly

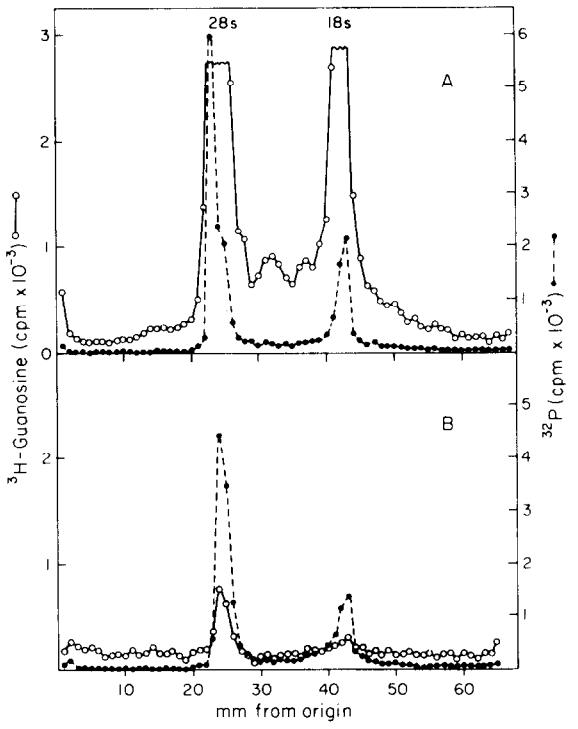

Fig. 1. Polysomal RNA's from control and FUR treated cells. CEF were grown to constant specific activity in medium containing ^{32}P(1 μCi/ml). When constant specific activity of the cellular RNA's had been achieved, the medium was replaced with medium containing [^{3}H]guanosine (5 μCi/ml. 1 μg/ml). In addition the medium placed on the treated cells contained FUR 20 mg/ml. After 4 hr incubation polysomes were collected, the RNA extracted, and analyzed by electrophoresis on columns of polyacrylamide. A) Control; B) FUR treated.

Table 2
RSV production in the presence of FUR.

A [^{3}H]leucine incorporated (cpm × 10^3)	10 hr	24 hr	48 hr	72 hr
Control	50	104	220	320
FUR-treated	44	100	206	280
B [^{3}H]adenosine incorporated (cpm × 10^3)	**8 hr**	**22 hr**	**48 hr**	**72 hr**
Control	2	8	24	30
FUR	2	11	28	35

A. Growing transformed cultures (2×10^6 cells per 100 mm petri dish) were transferred to fresh medium containing a concentration of amino acid one tenth of that normally present in Eagle's medium. One pair of cultures received in addition FUR (10 μg/ml), and another pair served as controls. After 30 min both cultures received [^{3}H]leucine (50 μCi /ml). At the indicated times thereafter the medium was removed, and replaced by fresh medium of identical composition. Virus was then purified in the normal way and pooled virus peak assayed for TCA precipitable radioactivity.
B. The conditions of the experiment were identical to those given for part A. However, the medium contained the normal concentrations of amino acids, and [^{3}H]adenosine (20 μCi/ml), 3 μg/ml) served as the radioactive precursor.

Table 3
Effect of FUR on RSV production.

Time (hr)	Control	FUR (10 μg/ml)
24	100%	5.5%
48	100%	2.6%
72	100%	3.7%

At 24, 48 and 72 hr after the addition of FUR to the culture medium samples were taken and titered for focus forming units. The results are presented as percent of control sample. The titers of the control samples ranged from 7.5×10^6 to 2×10^6 FFU/ml.

formed 4 S RNA and no detectable polysomal RNA (fig. 1). This pattern of inhibition of RNA synthesis is similar for both CEF and CEF-RSV.

When cultures of fully transformed CEF are monitored for virus production after treatment with FUR, they are found to produce virus at rates almost equal to control cultures for at least 3 days (table 2). This can be observed by incorporation of both protein and RNA precursors (table 2). Although the virus produced in the presence of FUR contains 70 S RNA [13], these virions are not as infective as those found in control cultures (table 3). This is probably due to a combination of two factors. The first is that FUR is most likely incorporated into the viral RNA and thereby inactivates a certain proportion of the virions. While this has not been rigorously established by following the incorporation of radioactive FUR, the aglycone, fluorouracil, is readily incorporated into viral RNA. Since the incorporated form in presumably FUMP, it seems reasonable to expect that FUR is incorporated as well. A second reason for the lowered infectivity of virus is that the glycosylation of the three viral glycoproteins is reduced in FUR-treated cells. After a 24-hour exposure to FUR the incorporation of glycosamine into the virus is reduced by more than 80% [13]. Since the viral glycoproteins are necessary for virus infectivity [17], the decreased glycosylation could account for the drop in focus forming titre in drug-treated cultures (table 3).

4. Discussion

These results indicate that under conditions where ribosomal RNA is neither processed nor made, RSV-RNA is made in normal amounts. The production of this viral RNA correlates with the production of both nuclear heterogeneous RNA and mRNA in CEF-RSV. The synthesis of mRNA in the presence of FUR has been substantiated by examining the production of mRNA or specific proteins in three independent cell systems: these are i) the production of virus-specific RNA in 3T3 cells transformed by SV-40 [18]; ii) the growth of vaccinia virus in L cells [19]; and iii) the differentiation of chick myoblasts and the synthesis of muscle proteins associated

with differentiation [20]. In all of these cases, FUR did not inhibit the expression of specific genetic functions in contrast to compounds such as actinomycin and 5-bromotubercidin that are known to block these and other processes dependent on continued mRNA production [13, 18, 20]. We propose, therefore, that the RSV-RNA is synthesized with a specificity that resembles that of mRNA and differs from that of rRNA.

While treatment of transformed cells with FUR does not affect the synthesis of viral RNA, there is a profound inhibition of the glycosylation of the viral glycoproteins that are located on the outer surface of the virion. In this respect FUR-virus resembles that produced in c/o′ cells (RSV) as described by Scheele and Hanafusa [21]. Further experiments can be expected to elucidate the mechanism by which FUR blocks the glycosylation of viral proteins.

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