The Effect of Temperature on the Synthesis of Rhinovirus Type 2 RNA

By R. A. KILLINGTON,* E. J. STOTT† AND D. LEE‡

Clinical Research Centre, Watford Road, Harrow, U.K.

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SUMMARY

The reduced yields of rhinovirus type 2 at temperatures above 37 °C were shown to result from the degradation of virus-induced RNA, leaving little RNA available for inclusion into mature infectious virions. The degradation occurred about 6 h p.i., and appeared to be selectively effecting the single-stranded species. Lysosomal nucleases do not appear to have a role in this supra-optimal degradation.

INTRODUCTION

The optimum temperature for isolation of rhinoviruses in general is 33 °C (Tyrrell & Parsons, 1960) and this is the optimum for the growth of rhinovirus type 2 (Stott & Heath, 1970). The yield of infectious virus at 39 °C is 400-fold lower than at 33 °C and the temperature-sensitive event appears to occur late in the growth cycle (Stott & Heath, 1970). Temperature sensitive mutants of viruses of many other groups have been studied and limitation of many different functions has been found to be responsible for the lower virus yield at supra-optimal temperatures (Lwoff, 1962): adsorption defects, inability to make either virus-specific nucleic acid or proteins, and maturation defects are examples. Rhinovirus type 2 shows neither adsorption nor uncoating defects at 39 °C (Stott & Heath, 1970) and in vitro studies suggest that its RNA-dependent RNA polymerase is as effective at 37 °C as at 33 °C (Yin & Knight, 1972). We therefore wished to examine what determined its natural temperature sensitivity.

This paper describes the effect of supra-optimal temperatures on the synthesis of acid-precipitable rhinovirus-specific RNA and its relation to the respective virus yields.

METHODS

Cells. L132 cells (Davies, 1960) or rhinovirus-sensitive HeLa cells (obtained from Dr V. V. Hamparian, Ohio State University) were grown in monolayers in rolled Winchester (2·5 l) bottles incubated at 36·5 °C in Eagle's basal medium (BM) containing 10% calf serum (Biocult Laboratories). L132 cells were removed from the glass with 0·125% (W/V) trypsin in PBS containing 0·025% (w/v) EDTA. HeLa cells were removed with 0·05% EDTA in PBS.

Single-cell suspensions of L132 cells were made at concentrations of 2 × 10⁶ cells/ml in BM, 2% calf serum, 5% tryptose phosphate broth, MgCl₂ (30 mm) and HEPES buffer pH 7·3 (MM). Suspensions were incubated for up to 12 h prior to virus inoculation and were

* Present address: Department of Microbiology, University of Leeds, Leeds LS2 9JT.
† Present address: Institute for Research on Animal Disease, Compton, Newbury, Berks.
‡ Present address: Department of Surgery, University of Otago, Dunedin, New Zealand.
stirred by magnetic bars in 150 ml round bottles submerged in a water bath at the required temperature.

**Virus strain and assay.** The HGP strain of rhinovirus type 2 was isolated at the Common Cold Unit and purified by three successive terminal dilutions. Stock virus was grown to high titre (10⁹ p.f.u./ml) in Winchester roller bottles of HeLa cells at 33 °C. Virus was assayed by the method of Stott & Heath (1970).

**Virus growth experiments and labelling conditions.** Following a 12 h incubation period, stirred suspensions of cells were centrifuged and resuspended in a small volume of virus to give an input multiplicity of approx. 10 p.f.u./cell, unless otherwise stated. Virus was allowed to absorb for 30 min at 33 °C, the cells were then washed three times in cold MM, resuspended in warm MM containing 5 μg/ml actinomycin D (a gift of Merck, Sharpe and Dohme) at a final concentration of 2 × 10⁶ cells/ml and incubated at the appropriate temperature in a water bath, the temperature of which varied by < 0.2 °C. The moment at which reincubation began was taken as zero time. Actinomycin D at 5 μg/ml inhibited more than 99% of the incorporation of ³H-uridine into host cell RNA. One h later suspensions were supplemented with 5-³H-uridine (> 100 000 mCi/mmol, Radiochemical Centre, Amersham) at a concentration of 2 μCi/10⁶ cells. At various times after infection 0.2 ml samples were taken from the suspension, diluted in 1.8 ml MM and stored at −70 °C for future plaque assay, unless used immediately for infectious centre assay. At the same times, samples of 5 × 10⁶ cells were taken and assayed for acid precipitable ³H activity.

**Assay of ³H acid-precipitable RNA.** Unless otherwise stated the whole procedure was carried out in the cold, using cold chemicals. Samples of 5 × 10⁶ cells were centrifuged at 2000 g for 10 min and washed twice in isotonic saline; the final pellet was resuspended in 2 ml distilled water and left overnight at −70 °C. On thawing the RNA was precipitated by the addition of an equal vol. of 0.5 M-perchloric acid, and washed twice with 5% trichloroacetic acid (TCA). The final pellet was taken up in 1 ml N-sodium hydroxide, 1 ml distilled water, 3 ml 0.1% sodium pyrophosphate and 10 ml 10% TCA. The precipitates were filtered through discs of Whatman GF/A glass fibre paper, the paper was dried and submerged in Triton X-100/toluene (2:1 ratio), PPO and POPOP scintillation cocktail and ³H activity was counted in a Packard Tricarb scintillation counter.

Guanidine HCl (Sigma Limited) was used at a concentration of 3 mM.

**Extraction and fractionation of virus-induced ³H-labelled RNA.** At an appropriate time p.i. samples of 5 × 10⁷ cells were centrifuged at 2000 g for 10 min and resuspended in 0.1 M-acetate-0.1% SDS, pH 5.0 (AC/SDS), and the RNA doubly extracted with phenol. The aqueous layer was mixed with 2 vol. of cold ethanol and stored overnight at −20 °C. The resulting precipitate was centrifuged at 2000 g for 10 min, resuspended in 1 ml of AC/SDS and run through a Sephadex G-25 column. The appropriate fractions were layered on to 5 to 25% sucrose gradients (in AC/SDS) and centrifuged at 18 000 rev/min for 18 h in a 3 × 25 swing-out rotor of an MSE 65 ultracentrifuge. Samples were collected from the gradient via the bottom, using the principle of downward displacement. The samples were monitored for E₂₈₀ and ³H activity using the above scintillation cocktail.

**Lysosomal studies**

**Disruption of cells.** Samples of 8 × 10⁷ suspended cells were cooled at 4 °C and the cells harvested by centrifugation (at 5000 g for 2.5 min). The supernatant fluids were decanted and the cells washed once with 0.25 M-sucrose. The cells were then disrupted by exposure to pH 11.0 for 2 min at 0 °C. During disruption and in all subsequent procedures the concentration of sucrose was always above 0.25 M (Lee, 1970).
Temperature and rhinovirus RNA synthesis

Fig. 1. Effect of supra-optimal temperature on the growth of rhinovirus 2 in L132 cells.

○—○, 33 °C; △—△, 35 °C; ▲—▲, 37 °C; ■—■, 39 °C.

**Determination of latency and enzyme activity.** Acid phosphatase (orthophosphoric monoester phosphohydrolase—EC. 3.1.3.2) was assayed in triplicate at pH 4.75, using glycerophosphate (Grade I, Sigma Ltd) as substrate (Lee, 1970). Specific activity was expressed in milli units (mU)/mg protein.

The latency of the enzyme is defined as the percentage of the total activity present in the homogenate which was unmasked by incorporation of 0.05% Triton X-100 (Sigma Ltd) into the reaction mixture. Consequently, the latency value gives an indication of the extent to which the lysosomes remained intact.

**RESULTS**

**Virus growth and RNA synthesis at different temperatures**

Following the addition of $^3$H-uridine, samples were taken at hourly intervals from infected cell suspensions incubated over a range of temperatures and assayed for virus infectivity and acid-precipitable RNA ($^3$H activity). At the optimum temperature (33 °C) the maximum virus yield was reached by 9 h after infection, whereas at the raised temperatures the yields were lower and were attained earlier (Fig. 1). At 39 °C the virus yield was 400-fold lower than at 33 °C. Monitoring of precipitable $^3$H activity indicated a good correlation between the rate of RNA synthesis and the production of infectious virus (Fig. 2). The significant feature of these experiments was the decline in acid-precipitable RNA at 6 h p.i. following incubation at and above 37 °C (Fig. 3). At 39 °C little virus-specific RNA was made in infected cells.
Fig. 2. Correlation of virus-induced RNA (precipitable) with infectious particle formation (p.f.u./ml) at 33°C and 39°C for rhinovirus 2. At 33°C: ●—●, RNA; ○--○, p.f.u./ml. At 39°C: ▲--▲, RNA; ▲--▲, p.f.u./ml.

Fig. 3. Virus-induced RNA (precipitable) following incubation of rhinovirus 2 at 33°C (●—●), 37.5°C (▲—▲) and 38.5°C (■—■).
Effect of temperature shift on virus growth and RNA synthesis

The fate of the virus-specific RNA was examined further by shifting infected cell suspensions from 33 °C to 39 °C at various intervals, and assaying for acid-precipitable RNA (Fig. 4). Infected cells, shifted up to 39 °C at times up to 5 h p.i., supported RNA synthesis as normal until about 6 h p.i. when the 3H count in acid-precipitable RNA decreased. Infected cells, shifted from 33 °C to 39 °C at 6 h or more p.i., showed an immediate fall in the precipitable 3H activity at the higher temperature.

These experiments suggested that incubation at supra-optimal temperatures resulted in normal RNA synthesis until approx. 6 h p.i. when an apparent degradation of pre-formed RNA occurred.

Effect of guanidine-HCl on RNA synthesis

Guanidine-HCl, which has been shown to inhibit the synthesis of poliovirus RNA (Crowther & Melnick, 1961; Baltimore et al. 1963), also inhibited rhinovirus RNA synthesis and was used here in order to examine the effect of temperature on the degradation of pre-formed RNA, with de novo synthesis at a minimal rate (Fig. 5). An infectious cycle was initiated at 33 °C and at 5 and 6 h p.i. the cells were divided into three samples (a, b, and c), two of which (b and c) received guanidine HCl; samples a and b were left at 33 °C whereas sample c was transferred to 39 °C. The procedure was repeated at 6 h p.i. Under these conditions there is little degradation of RNA at 33 °C whereas at the higher temperature up to 50% of pre-formed virus RNA is degraded.
Fig. 5. Effect of adding guanidine-HCl (3 mM) and either shifting to 39 °C or keeping at 33 °C on virus-induced RNA (precipitable). Replicate cultures were given guanidine at times 5 h and 6.5 h p.i. and either transferred to 39 °C or kept at 33 °C: \(\blacksquare\) -- \(\blacksquare\), 33 °C throughout no guanidine; 33 °C and guanidine: \(\square\) -- \(\square\), 5 h; \(\blacksquare\) -- \(\blacksquare\), 6.5 h; 39 °C and guanidine: \(\Delta\) -- \(\Delta\), 5 h; \(\blacksquare\) -- \(\blacksquare\), 6.5 h.

Virus specificity of the effect

Degradation of virus-specific RNA could result from the general effect of a higher temperature on the metabolism of the normal host cell and not be the result of a specific virus-cell interaction. This was examined by observing the pattern of virus RNA synthesis in cells previously incubated at 39 °C for 6 h prior to absorption. Cells infected in this way were kept at 39 °C or transferred to 33 °C following infection and samples assayed at various time intervals for acid-precipitable RNA and virus infectivity. No differences were recorded between cells pre-incubated at 33 °C or 39 °C, suggesting that the RNA degradation is the result of a specific virus-cell interaction.

Effect of temperature-shift on RNA species of infected cells

Cells were infected at 33 °C and at 6 h p.i.; one half was transferred to 39 °C and the other half was left at 33 °C. Two h later RNA was extracted from the cells and analysed by velocity sedimentation on sucrose gradients (Fig. 6). In cells maintained at 33 °C, 66% of the radioactivity sedimented in a peak at 30 to 32S and 10% at 16S. In this respect the distribution differs little from a 6 h profile (unpublished observation). When cells had been shifted to 39 °C, 9% of radioactivity was found at 16S but only 19% sedimented at 30 to 32S, and 63% remained at the top of the gradient. Whereas 64% of the 16S material detected in cells maintained at 33 °C was still present after the shift to 39 °C, only 20% of
Temperature and rhinovirus RNA synthesis

Fig. 6. Effect of temperature shift on rhinovirus 2 induced RNA. Replicative cultures were either maintained at 33 °C (■) or moved to 39 °C (□) at 6 h after infection. RNA extracted from cells 2 h later was analysed on sucrose gradients. Arrows indicate the positions of 28S and 16S ribosomal RNA.

the 30 to 32 S RNA remained after incubation at 39 °C. These results indicate that after shifting to 39 °C most of single-stranded virus RNA is degraded to small mol. wt. material.

It appears, therefore that in rhinovirus infected cells, degradation of virus-specific RNA which occurs about 6 h p.i. limits the final yield of infectious progeny virus at 39 °C. These results are similar to those of Fiszman (Fiszman et al. 1970) using a ts mutant of poliovirus. Fiszman et al. (1970) suggested that lysosomal labilization leading to the release of nucleases could be the reason for the sudden degradation of virus RNA which occurred in cells infected with ts mutants and incubated at a supra-optimal temperature. This possibility was investigated for rhinovirus-infected cells, using the release of acid phosphatase as a marker for lysosomal lability.

Labilization of lysosomal membranes

An earlier report (Killington et al. 1974) has shown that lysosomes of cells incubated at 33 °C and infected with rhinovirus type 2 remain stable throughout the infection. Similar experiments were performed to study the release of lysosome-bound acid phosphatase in infected cells incubated at higher temperatures. Cells were infected and incubated at 33 °C; after 5, 6 and 7 h of incubation, samples were transferred from 33 °C to 39 °C and 1 h after transfer were assayed for acid phosphatase by the method described earlier (Killington et al. 1974). Further cells were infected and incubated at 39 °C for 9 h when they were assayed as above. In both experiments mock-infected cells were treated in a similar manner. These experiments were done in duplicate on separate occasions and in no sample did the acid phosphatase activity differ between (a) cells at 33 °C and cells at or transferred to 39 °C and (b) infected cells and mock-infected cells. The specific activity remained constant at 40 mU/mg protein, and the latency of acid phosphatase remained constant at 65%.
DISCUSSION

The ts event in the growth of rhinovirus type 2 at 'supra-optimal' temperatures appears to be related to the availability of virus-specific RNA for inclusion in mature infectious virions. Virus infected cells incubated at 39 °C for the whole of the growth cycle synthesize little virus-specific RNA, which results in a much lower titre of infectious virus. Virus infected cells incubated at 36 to 38 °C show an initial increase in acid precipitable RNA which falls off at about 6 h p.i. This fall off in available RNA was emphasised by the temperature shift experiments and is thought to result from an increase in the degradation of RNA and not merely a change in the synthesis/degradation ratio at the higher temperature as shown by the guanidine experiments.

RNA degradation is probably the result of nuclease action. Analyses on sucrose gradients show that in cells transferred to 39 °C 6 h p.i. 80% of the single-stranded RNA is degraded. The relative resistance of double-stranded RNA also explains the levelling off of the acid-precipitable degradation curve (Fig. 2), and the delay in RNA degradation until 6 h p.i., when the bulk of single-stranded ribonuclease sensitive RNA synthesis begins (Gaunt, 1973; R. A. Killington et al. unpublished observations).

Such a nuclease could be released by rupture of the lysosomal membrane at the supra-optimal temperatures, as is common in many virus-infected cells (Allison & Sandelin, 1963; Malluci & Allison, 1965; Flanagan, 1966). Although not as satisfactory as the measurement of nuclease activity, the experiments which investigated acid phosphatase activity suggested no such rupture. It seems reasonable to conclude that membrane changes which release lysosomal nucleases should allow the free flow of acid phosphatase.

The nuclease could well be a cytoplasmic enzyme triggered off by virus growth at supra-optimal temperatures, or possibly a dual role polymerase which in vivo acts as a nuclease/polymerase complex, with the former being greater at the higher temperatures.

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REFERENCES


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