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Temperature-sensitive Mutants of Equine Arteritis Virus

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SUMMARY

Seventeen temperature-sensitive mutants of equine arteritis virus, a non-arthropod-borne togavirus, have been isolated. 5-Fluorouracil, o-methylhydroxylamine and ethyl methanesulphonate were used as mutagens. The mutants were characterized by their ability to synthesize virus RNA and virus proteins at the permissive (35 °C) and restrictive temperature (40 °C) using autoradiography of cells labelled with ^{3}H-uridine in the presence of actinomycin D and immunofluorescence respectively. Among the mutants, four were unable to synthesize virus RNA and virus proteins at 40 °C (RNA$^-$/protein$^-$). The other mutants were RNA$^-$/protein$^+$ (3); RNA$^\pm$/protein$^-$ (2); RNA$^+$/protein$^+$ (6) and RNA$^+$/protein$^-$ (1).

INTRODUCTION

Equine arteritis virus (EAV) is the aetiological agent of a febrile disease of horses which is usually accompanied by oedema and petechiae; in pregnant mares the infection may induce abortion (Doll *et al.* 1957). EAV is a non-arthropod-borne member of the family *Togaviridae* (Horzinek, 1973) which contains four genera: alphavirus, flavivirus, rubivirus and pestivirus. Equine arteritis virus and lactic dehydrogenase virus are additional members of the family outside the existing genera (Porterfield *et al.* 1978). The genome of EAV is an infectious colinear, single stranded RNA molecule of mol. wt. about 4×10^6 (van der Zeijst *et al.* 1975). The virus has three structural polypeptides with mol. wt. of 12000 (VP1), 14000 (VP2) and 21000 (VP3) (Zeegers *et al.* 1976). Thus, only very little of the genetic information present in the virus RNA is used for the synthesis of the structural proteins of the virion. The aim of our work is to determine how these virus proteins are synthesized and ascertain the function of the remaining part of the virus genome. In the present paper we describe the isolation and preliminary characterization of temperature-sensitive (*ts*) mutants of EAV which will serve as a tool to support these studies.

METHODS

Cells. Monolayers of Vero and BHK21 cells were grown in Dulbecco's modification of Eagle's medium (DMEM) containing 10% foetal calf serum (FCS), 100 international units/ml penicillin and 100 μg/ml streptomycin.

Virus. A high titred stock of the Bucyrus strain of EAV (Doll *et al.* 1957) was prepared by infecting monolayers of Vero cells in 490 cm^2 roller flasks (Corning, N.Y., U.S.A.) with virus diluted in PBS-DEAE (PBS, containing 50 μg/ml DEAE-dextran, Pharmacia, Uppsala, Sweden) supplemented with 2% FCS to give an m.o.i. of 0·1. In all infection experiments the cells were pre-washed with PBS-DEAE and DEAE-dextran was also

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present in the inoculum since it is known to enhance the adsorption of EAV to cells (Hyllseth, 1969). After 1 h at 37 °C the inoculum was removed and replaced by 25 ml salt solution B containing 137 mM-NaCl, 4 mM-KCl, 1·6 mM-$CaCl_2$, 1 mM-$MgCl_2$, 23·8 mM-$NaHCO_3$, 10 mM-glucose and 20 mg/l of phenol red, pH 7·2. Virus was harvested 40 h after infection when most of the cells showed cytopathology. After removal of the debris by low speed centrifugation, virus was pelleted at 100000 *g* for 4 h and resuspended in 200 μl TES-buffer (0·02 M-tris-HCl, 1 mM-EDTA and 0·1 M-NaCl, pH 7·4) per flask. This suspension (containing about 10^9 p.f.u./ml) was frozen and stored at −70 °C and served as stock for all experiments.

Plaque assay. For virus cloning and isolation of mutants a plaque assay as described before (van der Zeijst *et al.* 1975) was employed, with the exceptions that Vero cells were used and that the overlay contained 0·6% agarose (BDH Chemicals Ltd, Poole, Dorset, England) in DMEM supplemented with 2% FCS. The plaques were visualized by staining the monolayers with 0·02% neutral red in PBS after 4 days.

A modified plaque assay was used to screen a large number of virus clones for temperature sensitivity. Vero cells (4×10^5 in 1 ml DMEM supplemented with 10% FCS) were mixed with 100 μl of virus dilutions and plated in the 19 mm squares of Sterilin replitrays (Sterilin, Teddington, Middlesex, England). After 4 h incubation at 40 °C the cells had formed a monolayer and were overlaid with 1·5% carboxymethylcellulose (low viscosity, BDH Chemicals, Ltd) in DMEM supplemented with 2% FCS. Four days later the overlay was removed and the cells were washed twice with PBS, fixed and stained with 0·75% crystal violet in 9% formaldehyde. Although infectivity titres were about tenfold lower than those obtained in the normal plaque assay this method proved faster and simpler to carry out.

Chemical mutagenesis

The following mutagens were used: ethyl methanesulphonate (EMS), an alkylating agent that modifies nucleic acids in various ways (Freese, 1963), o-methylhydroxylamine (MHA), hydroxylamine (HA) and the base analogue, 5-fluorouracil (5-FU). HA and MHA are mutagenic by their reaction with cytosine. HA also attacks uracil and can cause splitting of the pyrimidine ring; MHA lacks this effect (Kochetkov *et al.* 1963).

EMS. EAV was diluted to 10^6 p.f.u./ml in 0·3 M-phosphate buffer, pH 7·0, containing 90 mM-EMS (Merck-Schuchard, Darmstadt, Germany) and kept at 20 °C for 60 min. The sample was then diluted with PBS-DEAE containing 2% FCS and plaque titrated at 35 °C.

HA and MHA. EAV was diluted to 10^7 p.f.u./ml in a solution containing 1·25 M-HA (BDH Chemicals Ltd), 0·5 M-NaCl, 37·5 mM-phosphate buffer, 1 mM-$MgSO_4$ (pH 7·2) or in 1·25 M-MHA (ICN, K & K Laboratories Inc., Plainview N.Y., U.S.A.), 37·5 mM-phosphate buffer, 1 mM-$MgSO_4$, 30 mM-cysteine and 60 mM-dithiothreitol (pH 7·2), and kept at 20 °C for 60 min. After dilution with PBS-DEAE containing 2% FCS the samples were titrated at 35 °C.

5-FU. Vero cell monolayers were infected with EAV at an m.o.i. of about 20. After 2 h at 35 °C the inoculum was removed and replaced by 20 ml salt solution B in the presence of 8 mM-5-FU (Koch-Light Laboratories, Colnbrook, Bucks., England). Virus was harvested 19 h p.i.

Selection of mutants. Mutagenized stocks had infectivity titres that were 10 to 1000 times lower than those of untreated controls. Well-isolated plaques were punched out using the mouthpiece of a Pasteur pipette and transferred into Vero cell monolayer tube cultures. After incubation for an additional 4 days at 35 °C a c.p.e. was seen and the cell medium was collected and assayed for infectivity, both at the permissive and non-permissive

temperatures. When the plaque titre at 35 °C exceeded that obtained at 40 °C by a factor of 10^3 or more, the clone was considered a possible mutant. Such mutants were re-cloned and used for the preparation of working stocks by one further passage at 35 °C at a m.o.i. of 0·1.

Antisera. Anti-EAV serum was prepared by multiple inoculations of hamsters with crude extracts of EAV-infected (8 h at 40 °C) BHK21 cells. The cells were lysed in TES buffer containing 0·5% Triton X-100 and 0·5% naphthalenedisulphonate, and mixed with an equal volume Freund's complete adjuvant. Hamsters were injected intramuscularly with 0·2 ml of mixture corresponding to about 2×10^6 cells. The antiserum was adsorbed to BHK21 cells that had been fixed with ethanol–acetic acid (3:1 by vol.); it was able to precipitate the VP1 and VP2 proteins of EAV. FITC-labelled rabbit anti-hamster IgG was obtained from Miles-Yeda Ltd, Rehovot, Israel.

Virus RNA and protein synthesis. To exclude mutants defective in adsorption at 40 °C, BHK21 cells grown on 18 × 18 mm coverslips in 35 mm Petri dishes were infected at 35 °C, using an m.o.i. of about 100. After 1 h the inoculum was removed and replaced by 1 ml DMEM supplemented with 10% FCS and the cells were either kept at 35 °C or shifted to 40 °C. For the assay of virus RNA synthesis, the cells received 1 μg/ml actinomycin D (Serva, Heidelberg, Germany) in their growth medium 1 h p.i. The cells were labelled with 5-^{3}H-uridine (10 μCi/ml, 29 Ci/mmol, The Radiochemical Centre, Amersham, Bucks., England) from 2·5 to 8 h p.i. at 40 °C or from 2·5 to 20 h at 35 °C. At the end of this period the coverslips were washed once in PBS, twice in 5% trichloroacetic acid and once in ether–ethanol (1:1, by vol.). The dry coverslips were mounted on to microscope slides, dipped in NTB2 liquid photographic emulsion (Eastman-Kodak Co., Rochester, N.Y.) and exposed at 4 °C for 10 days. The slides were developed in Kodak D-19 and stained with Giemsa solution (Merck).

Virus protein synthesis was assayed by indirect immunofluorescence. Infected coverslip cultures were washed three times in PBS, dried and fixed with acetone at room temperature for 15 min. After incubation for 30 min with a 1:10 dilution of anti EAV serum, the coverslips were washed three times with PBS and the procedure repeated with FITC-labelled anti-hamster IgG serum. Finally, the coverslips were mounted on slides in PBS–glycerol (1:2 by vol.) and read in an epifluorescence microscope.

RESULTS

Selection of a permissive and a non-permissive temperature for ts-*mutants*

Wild-type EAV was plaque titrated on Vero cell monolayers in tissue culture flasks. The flasks were incubated in the dark, in waterbaths at intervals of 1 °C from 27 °C to 42 °C. Below 35 °C the cells did not grow well and no plaques were formed. Above 41 °C only a few plaques were observed. Between 35 and 40 °C the number of plaques was constant. Therefore, 35 and 40 °C were chosen as permissive and non-permissive temperatures, respectively, for the selection of possible mutants. Plaques at 40 °C were generally larger than those at 35 °C with much individual variation in size.

Mutagenesis of EAV and selection of mutants

From a total of 250 plaques treated with EMS only one was a *ts*-mutant. Before using HA or MHA as mutagens we have measured their virus inactivating potential. As expected, HA inactivated EAV more rapidly than MHA; both reactions followed first order kinetics with half-times of 3·8 min and 8·7 min, respectively. Of the 486 virus clones tested after MHA-treatment of EAV, 11 *ts*-mutants were obtained. In contrast, none of the 219

Table 1. *Properties of 17* ts *mutants of EAV**

Mutant	Mutagen used†	Virus titre (p.f.u./ml) at 35 °C	Virus titre (p.f.u./ml) at 40 °C	Reversion‡ frequency	RNA synthesis§ at 40 °C	Protein synthesis‖ at 40 °C
Wild-type	—	$1{\cdot}1 \times 10^{7}$	$0{\cdot}9 \times 10^{7}$	—	+	+
ts-6	MHA	$1{\cdot}8 \times 10^{7}$	$2{\cdot}0 \times 10^{3}$	$1{\cdot}1 \times 10^{-4}$	−	−
ts-21	EMS	$1{\cdot}4 \times 10^{7}$	$< 1{\cdot}0 \times 10^{4}$	$< 7{\cdot}1 \times 10^{-4}$	+	+
ts-27	5-FU	$1{\cdot}5 \times 10^{7}$	$< 1{\cdot}0 \times 10^{4}$	$< 6{\cdot}7 \times 10^{-4}$	−	+
ts-35	MHA	$1{\cdot}9 \times 10^{7}$	$1{\cdot}8 \times 10^{3}$	$9{\cdot}5 \times 10^{-4}$	±	−
ts-36	MHA	$6{\cdot}0 \times 10^{6}$	$1{\cdot}0 \times 10^{2}$	$1{\cdot}7 \times 10^{-5}$	−	−
ts-72	5-FU	$1{\cdot}5 \times 10^{7}$	$< 1{\cdot}0 \times 10^{4}$	$< 6{\cdot}7 \times 10^{-4}$	+	+
ts-75	MHA	$5{\cdot}0 \times 10^{8}$	$3{\cdot}0 \times 10^{4}$	$6{\cdot}0 \times 10^{-5}$	+	+
ts-79	MHA	$5{\cdot}0 \times 10^{7}$	$1{\cdot}0 \times 10^{3}$	$2{\cdot}0 \times 10^{-5}$	+	+
ts-86	5-FU	$3{\cdot}8 \times 10^{8}$	< 5	$< 1{\cdot}3 \times 10^{-8}$	−	+
ts-88	MHA	$1{\cdot}8 \times 10^{6}$	< 5	$< 2{\cdot}8 \times 10^{-6}$	not done	not done
ts-93	MHA	$9{\cdot}0 \times 10^{7}$	< 5	$< 5{\cdot}6 \times 10^{-8}$	+	+
ts-130	5-FU	$> 6{\cdot}0 \times 10^{7}$	$2{\cdot}5 \times 10^{4}$	$< 4{\cdot}2 \times 10^{-4}$	−	−
ts-172	MHA	$1{\cdot}0 \times 10^{8}$	$1{\cdot}0 \times 10^{2}$	$1{\cdot}0 \times 10^{-6}$	+	−
ts-228	MHA	$4{\cdot}0 \times 10^{7}$	$1{\cdot}0 \times 10^{2}$	$2{\cdot}5 \times 10^{-6}$	−	+
ts-254	MHA	$7{\cdot}0 \times 10^{7}$	$1{\cdot}0 \times 10^{4}$	$1{\cdot}4 \times 10^{-4}$	−	−
ts-275	5-FU	$1{\cdot}4 \times 10^{8}$	< 5	$< 3{\cdot}6 \times 10^{-8}$	±	−
ts-304	MHA	$9{\cdot}0 \times 10^{7}$	$1{\cdot}0 \times 10^{4}$	$1{\cdot}1 \times 10^{-4}$	+	+

* To obtain high enough titres, the virus was passed twice after re-cloning (see Methods).

† Ethyl methanesulphonate (EMS), 5-fluorouracil (5-FU) and o-methylhydroxylamine (MHA).

‡ The reversion frequency is the ratio of the plaque titre measured at 40 °C (back mutants) to the virus titre measured at 35 °C.

§ Determined by autoradiography of infected cells, labelled with ^{3}H-uridine in the presence of actinomycin D.

‖ Assayed by immunofluorescence.

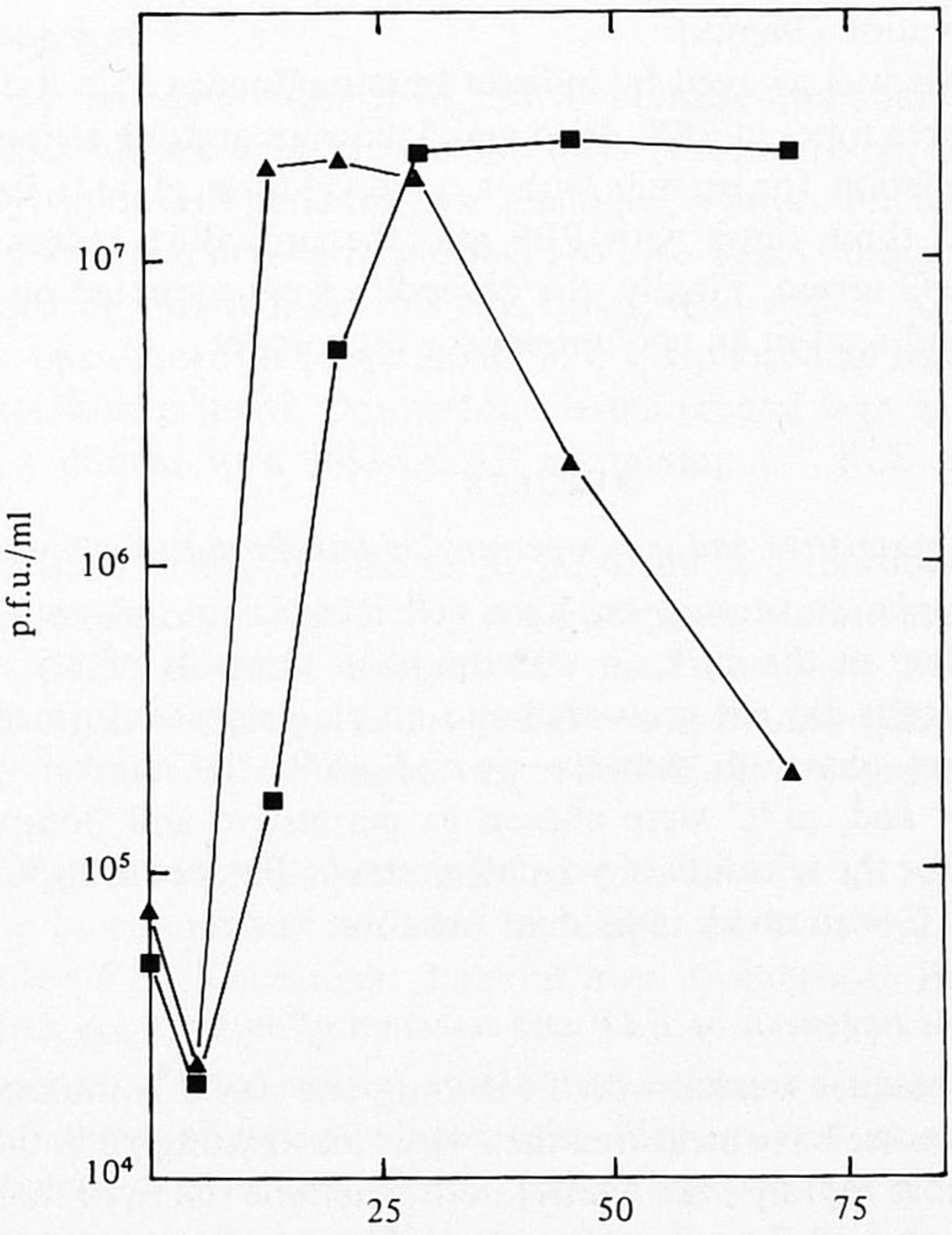

Fig. 1. Growth of EAV at 35 °C (■—■) and 40 °C (▲—▲).
Cells were infected at 50 p.f.u./cell.

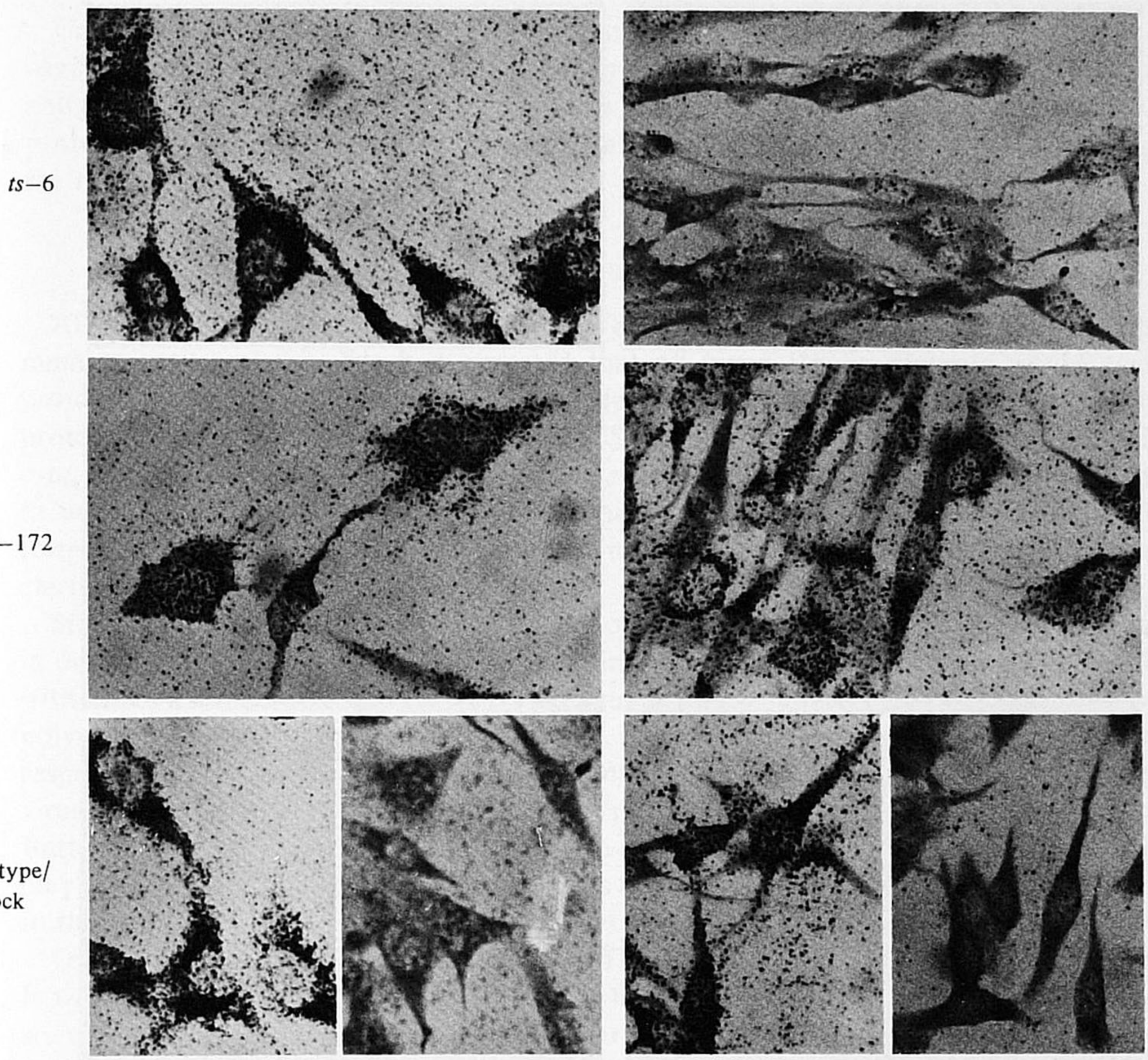

Fig. 2. Autoradiographs of BHK21 cells infected with wild-type EAV or *ts* mutants and labelled with ^{3}H-uridine in the presence of actinomycin D. Mock-infected and wild-type infected cells at 35 and 40 °C and representatives of RNA^{-} mutants (*ts*-6) and RNA^{+} mutants (*ts*-172) at either temperature are shown.

plaques tested after HA mutagen treatment was thermosensitive. A total of 353 isolated plaques from EAV grown in the presence of 5-FU was tested; five were shown to be *ts*-mutants.

In all we have examined 1308 virus clones from mutagenized EAV stocks. Among these were 17 *ts*-mutants which grew as well as wild-type EAV at 35 °C and gave 10^{-3} or less revertants at 40 °C. A number of properties of these mutants are summarized in Table 1.

RNA and protein synthesis of mutants at the restrictive temperature

The mutants were studied for their ability to synthesize virus RNA and proteins at the permissive and restrictive temperature. As shown in Fig. 1, maximum yields of wild-type EAV at 40 and 35 °C are reached at 13 and 30 h p.i., respectively. Therefore we assayed the mutants for intracellular virus RNA and proteins at 8 h p.i. at 40 °C and at 20 h at 35 °C.

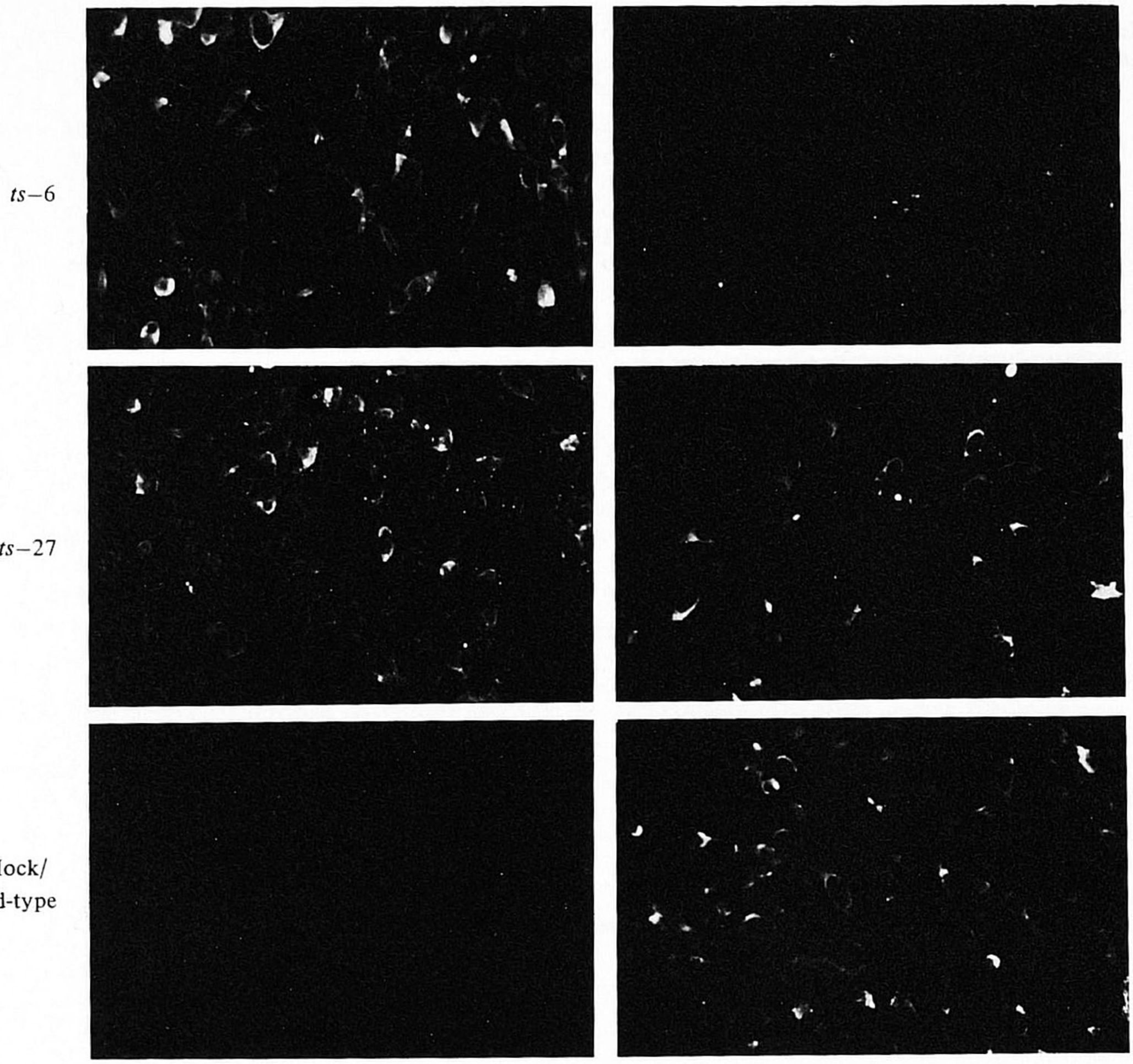

Fig. 3. Immunofluorescence of BHK21 cells infected with wild-type EAV or *ts* mutants. There are two classes of mutants: protein$^+$ mutants (e.g. *ts*-27), synthesizing virus antigens both at 35 and 40 °C and protein$^-$ mutants (e.g. *ts*-6) that do not produce virus proteins at 40 °C. Wild-type virus-infected cells (at 40 °C) and mock-infected cells (at 35 °C) are shown as controls.

RNA synthesis

Virus RNA synthesis was measured by labelling infected cells with ^{3}H-uridine in the presence of actinomycin D. Cultures infected with either wild-type or mutant virus incorporated about the same amount of ^{3}H-uridine at 35 °C (60000 ct/min per 18 × 18 mm coverslip, with a background of 7000 ct/min in uninfected cultures). At 40 °C, however, the incorporation levels for most mutants were lower than the level for wild-type virus (50000 ct/min per coverslip, 4000 ct/min background). When stimulation was only two- to threefold above the background in mock-infected cells it was difficult to score mutants as RNA$^+$ or RNA$^-$.

Using autoradiography it appeared that at 35 °C most cells became infected by both wild-type virus and by mutants. Cells infected with seven mutants were labelled at 35 °C but not at 40 °C. These mutants were scored as RNA$^-$ (e.g. *ts*-6, Fig. 2). Another seven mutants were RNA$^+$. They were labelled equally as well as wild-type infected cells at both temperatures (e.g. *ts*-172, Fig. 2). Two mutants were scored as RNA$^\pm$ because only a small number of grains were visible in the infected cells. The results are summarized in Table 1.

Protein synthesis

Cells infected at 35 °C with various *ts* mutants and kept at 35 °C or shifted to 40 °C were assayed for EAV-proteins by immunofluorescence. All mutants produced virus antigens at 35 °C. However, seven mutants (e.g. *ts*-6, Fig. 3) did not synthesize virus proteins at 40 °C whereas nine mutants were unaffected (e.g. *ts*-27, Fig. 3). Table 1 gives the results for all mutants.

DISCUSSION

All four possible combinations between RNA$^-$ or RNA$^+$ and protein$^-$ or protein$^+$ mutants were isolated. We had expected that all seven RNA$^-$ mutants would also be protein$^-$, because they probably have a *ts*-lesion in one or more of the non-structural proteins involved in virus mRNA synthesis (Strauss *et al.* 1976; Pfefferkorn, 1977). However, three RNA$^-$ mutants (*ts*-27, *ts*-86 and *ts*-228) are protein$^+$. This is at present difficult to understand. One explanation could be that RNA synthesis at 40 °C is only partially restricted, still allowing for the synthesis of mRNA. More experiments will be needed to clarify this point.

Six out of the seven RNA$^+$ mutants are also protein$^+$. These mutants could have a defect in one of the virus structural proteins, resulting in impaired virus assembly (compare, e.g., with *ts*-23 of Sindbis virus; Yin, 1969; Smith & Brown, 1977) or, alternatively, virus polyproteins could be synthesized that are not recognized by the proteolytic enzymes responsible for processing. Several *ts* mutants of two other togaviruses, Semliki Forest virus and Sindbis virus, accumulate protein precursors at the restrictive temperature (see Butterworth, 1977). We have no evidence so far that cleavage of protein precursors does play a role in the replication of EAV. There is ample evidence, however, for this mechanism in the replication of many other animal RNA viruses (Butterworth, 1977).

One RNA$^+$ mutant, *ts*-172, is protein$^-$. This mutant deserves a more detailed study. It would be of particular interest to know whether virus mRNA is made at 40 °C and, if so, what products this RNA is able to code for. A similar type of mutant has been described for Semliki Forest virus and for Eastern equine encephalitis virus (Tan *et al.* 1969; Zebovitz & Brown, 1970).

There are at least three mutants (*ts*-86, *ts*-93 and *ts*-275) with a very low reversion frequency (10^{-8} or 10^{-7}). In fact, the selection for this type of mutant could be a feature of our isolation procedure. First, EAV only produces plaques over a relatively small temperature range and second, it proved impossible to elute EAV efficiently (< 10 p.f.u./plaque) from isolated plaques, as can be done easily for other viruses. Therefore an additional multiplication step had to be inserted with the risk of accumulating revertants before the progeny virus of a plaque could be tested for its *ts* character. Thus we may have selected for mutants containing multiple *ts* lesions.

EMS, HA, MHA and 5-FU were used as mutagens. The best results were obtained with MHA and 5-FU, which gave 2·1% and 1·5% mutants, respectively. EMS only gave 0·4%, whereas no mutants ($< 0{\cdot}5\%$) were isolated using HA. To our knowledge MHA has not been used before for mutagenesis of animal viruses. Our results suggest that it should be applied more widely.

In the togavirus family, complementation has been found so far only in two series of mutants, both derived from the HR strain of Sindbis virus (Burge & Pfefferkorn, 1966; Strauss *et al.* 1976). No complementation could be demonstrated in another series of *ts* mutants of Sindbis virus or between *ts* mutants of Semliki Forest virus (Tan, 1969; Atkins *et al.* 1974). Virus yields for Sindbis virus are 10^3 to 10^4 p.f.u./cell and although

complementation of mutants resulted in only 1 to 2% of the yield at the permissive temperature, it could be easily demonstrated (Burge & Pfefferkorn, 1966). It will be interesting to see whether EAV mutants can complement each other. However, in the case of EAV, where virus yields are usually only 100 p.f.u./cell, complementation probably would be detected only when it is very efficient. A start could be made by testing some mutants that are likely to complement each other, e.g. RNA^+ and RNA^- mutants.

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