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Temperature-sensitive mutants of mouse hepatitis virus type 3 (MHV-3): isolation, biochemical and genetic characterization

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Summary. Mouse hepatitis virus 3 (MHV-3) is highly hepatotropic in sensitive mice. Temperature-sensitive mutants (ts mutants) induced by N-methyl-N′-nitrosoguanidine and 5-fluorouracil were isolated. Twelve mutants which were able to induce the formation of syncytia at 33 °C but not at the restrictive temperature (39.5 °C) were selected for detailed study. No viral RNA synthesis was detected after infection at the restrictive temperature with six of the mutants (RNA^-) whereas six others were RNA^+, although they displayed RNA synthesis which was generally reduced. No differences have been detected in the size of the genome or the viral-intracellular RNA species found in wild type virus or ts mutant infected cells at permissive temperature. The pattern of virus-induced proteins analyzed after immunoprecipitation by SDS-PAGE was similar in wild type virus and RNA^+ mutant infected cells at 39.5 °C. Complementation experiments between ts mutants enabled us to distinguish five groups. Three of the groups contained RNA^- mutants and two of them RNA^+. Plaques made by mutants in one group displayed characteristic features that distinguished them from the wild type.

Introduction

Mouse hepatitis virus 3 (MHV-3), a member of the Coronaviridae, is an enveloped virus that contains a non-segmented, single-stranded, positivesense RNA genome of molecular weight 5.4×10^6 [11, 29]. The first step in MHV-3 RNA replication is the transcription of the genomic RNA into a full-length negative-stranded RNA [14]. This RNA is then transcribed into seven virus-specific mRNAs, the largest being the intracellular genomic form, and the others being the subgenomic mRNAs [5, 13, 15, 23, 25, 33]. These mRNAs have a "nested-set" structure such that all share identical sequences as the 3′ end [22]. Various strains of MHV cause different diseases in mice depending upon the particular race, age and immune status of the animals [30]. Although the genetic

structures of MHV-3 and MHV A 59 are similar [12], only the former is highly hepatotropic. Accordingly, MHV-3 induces a fatal hepatic necrosis in susceptible mice, the characteristic feature being the destruction of the hepatocytes [8]. Before this takes place the virus interacts with the sinusoidal barrier: i.e. with Kupffer and endothelial cells [19, 24].

Temperature-sensitive mutants have been isolated from MHV JHM [7, 31] or MHV A 59 [9, 20] but not from MHV-3. In this report we describe the isolation and characterization of 12 MHV-3 ts-mutants grouped into five complementation groups, three RNA^- and two RNA^+.

Materials and methods

Cells and virus

Mouse L 929, DBT and L_2 cells were grown separately in minimum essential medium (MEM) with glutamine containing 10% fetal calf serum. MHV-3 was a clonal isolate produced in L 929 cell cultures. For titration, viral suspensions were assayed on L 929 or L 2 cells cultured in 96-well microtiter plates [15].

Mutagenesis

1.2×10^6 L 929 cells in 35 mm tissue culture dishes were infected with 1 ml of 7×10^4 PFU/ml double cloned MHV-3. One hour after infection the inoculum was removed and replaced by 2 ml of MEM 5% FCS, 1% Bacto agar and either N-methyl-N′-nitrosoguanidine at 5 µg/ml or 5-fluorouracil at 150 µg/ml. After a 40 hr incubation at 33 °C the virus from the mutagenized cultures was plated on dishes that were stained with 0.1% neutral red 24 hrs later. Isolated plaques were then taken up with a Pasteur pipette, diluted in 300 µl PBS buffer and used to infect cells in 96-well microtiter plates at three different temperatures, namely 33 °C, 37 °C, 39.5 °C. After a 48 hr incubation virus clones that caused formation of syncytia at 33 °C or 37 °C, but not at 39.5 °C, were considered as potential ts mutants. Each clone was subcloned and tested to confirm its ts-nature. The ratio of the efficiency of plating at 39.5 °C to that at 33 °C was determined for each mutant. Only those mutants having a ratio equal to or lower than 5×10^{-2} were used in subsequent experiments.

Complementation assays

Complementation tests were performed in 96-well plates with L 929 cells seeded at 5×10^4 cells/well. The cells were infected with a 200 µl mixture of the two mutants or with individual mutants using a 5×10^4 PFU for each mutant. Virus adsorption was carried out at 39.5 °C for 1 hr. The inoculum was removed and fresh medium was added between 3 hrs and 24 hrs. Infections were terminated by freezing at −80 °C and infected cell lysates were subsequently titrated on L 2 cells at 33 °C and 39.5 °C. The complementation indexes were calculated using the formula of Fields and Joklik [6]:

$$CI = \frac{(A \times B)\,33\,^\circ C - (A \times B)\,39.5\,^\circ C}{A\,33\,^\circ C + B\,33\,^\circ C}$$

A CI greater than 3 was taken to indicate that two mutants belonged to different complementation groups, and a CI lower than 3 to the same complementation group.

Analysis of virus-specific RNA synthesis at 33 °C, 37 °C, and 39.5 °C

DBT cells in 24-well plates (5×10^4 cells/well) were infected with 200 µl of wild type or mutant viruses at 10^5 PFU/ml. After adsorption for one hour the virus was removed,

medium was added and infected cells were incubated at the desired temperature. The kinetics of incorporation were determined by adding actinomycin D (2 μm/ml) and ^{3}H uridine (10 μCi/ml, 27 Ci/mmol C.E.A.) 2 hours and 1 hour respectively before cell collection. For this, the medium was removed, the cells washed twice with PBS and then lysed in 300 μl of 1% SDS; 50 μl from each sample were assayed for acid-precipitable radioactivity.

Radiolabelling and purification of virus genomic RNA [13]

After 1 h adsorption at 0.5 PFU/cell the inoculum was removed and MEM was added. 5 hrs later the medium was replaced with phosphate-free MEM containing 1% dialysed foetal calf serum (FCS) and 50 μCi/ml of ^{32}P orthophosphate (Amersham). Supernatant fluids were collected 10 hrs later and cleared of cell debris by centrifugation at 15,000 **g** for 30 min at 4 °C. The supernatants were placed on 2 cushions consisting of 30 and 50% w) sucrose in NTE buffer (100 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA) and centrifuged at 115,000 **g** for 2.5 hrs. The virus band at the inferface between the cushions was collected and diluted threefold with NTE buffer. The ^{32}P labelled RNA was extracted at 4 °C in 1% SDS with phenol followed by phenol-choroform (1 : 1). The RNA was precipitated by the addition of 2.5 volumes of ethanol at —20 °C overnight, collected by centrifugation at 17,000 **g** for 10 min at 4 °C, dissolved in NTE buffer and centrifuged on linear 10–25% w/w sucrose gradients at 150,000 **g** for 1 hr. The radioactive RNA recovered in the peak was diluted in NTE and precipited by ethanol at —20 °C.

Preparation of intracellular virus-specific RNA [18]

After adsorption of the virus, 80% MEM without phosphate 20% MEM and 2% FCS were added for 90 min. Then infected cells were further incubated for 90 min in 90% phosphate-free MEM, 10% MEM, 2% dialysed FCS and 2 μg/ml actinomycin D and finally 100% of phosphate-free medium, 2% dialysed FCS, actinomycin D and 50 μCi/ml ^{32}P orthophosphate until 6–8 hrs p.i. The cell monolayers were washed with SS buffer (10 mM Tris-HCl pH 8.5, 60 mM NaCl, 1 mM EDTA). The cells were lysed in TKM buffer (10 mM Tris-HCl pH 7.2, 150 mM KCl, 10 mM $MgCl_2$) containing 0.5% triton N-101 and the nuclei were removed by centrifugation at 1,800 g for 5 min. The supernatant was adjusted to contain 1% SDS and was extracted with phenol followed by phenol-chloroform (1 : 1). After ethanol precipitation the RNAs were analysed by agarose gel electrophoresis.

RNA dot blot analyses

30 μg of unlabelled intracellular RNAs obtained from cells infected for 4 hrs at 39.5 °C were analysed by hybridization after immobilization on nitrocellulose paper (Schleicher and Schull). RNA was denatured by glyoxal-DMSO treatment at 50 °C for 1 hr and applied to nitrocellulose paper following the procedure described by Thomas [27]. The prehybridization and hybridization were performed in 50% de-ionised formamide, 5 × Denhardt's (1 × Denhardt's buffer contains 0.02% each of bovine serum albumin, Ficoll and polyvinyl-pyrrolidone), 5 × SSC (1 × SSC contains 0.15 M NaCl, 0.015 M Na citrate), 150 μg/ml salmon sperm DNA, 0.1% SDS, 10% Dextran sulphate. The prehybridization was performed at 42 °C for 12 hrs and hybridization at 42 °C for 36 hrs. The blots were washed four times at room temperature in 2 × SSC and and 0.2% SDS, twice in 2 × SSC, 0.1% SDS at 55 °C and exposed to Curix film (Agfa Gevaert) at —80 °C. The probes used in these analyses were either purified denatured ^{32}P genomic RNA of MHV-3, or ^{32}P "sense" RNA obtained by transcription of the E 1 gene cloned in a T 7 transcription vector (provided by R. Baric, University of North Carolina, Chapel Hill).

Labelling of intracellular proteins

DBT cells were infected at 33 °C, 37 °C, and 39.5 °C in 24-well plates with wild type or ts-mutants at a m.o.i. of 0.1 in MEM. MEM was replaced after 8 h by MEM without methionine and 30 min later ^{35}S methionine (specific activity: 1,000 Ci/m mol; Amersham) or ^{32}P orthophosphate (Amersham) were added at 15 μC/ml or 100 μC/ml respectively until 9 hrs p.i. After the labelling period, cells were washed twice with cold PBS and prepared for radioimmunoprecipitation. For pulse-chase experiments, after 30 min of labeling, fresh MEM was added to the infected cells until 11 hrs p.i.

Cells were lysed for 20 min at room temperature in L buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP40, 500 units aprotinin and 0.2 mg phenylmethyl-sulphonyl fluoride/ml) and centrifuged at 2,000 g for 10 min [32]. Soluble proteins were obtained after centrifugation at 100,000 **g** for 90 min.

Immunoprecipitation was accomplished with protein A-Sepharose CL-4 B (Pharmacia) as the solid phase absorbant. To 200 μl of the labelled infected or control cell extracts, 2 μl of polyclonal MHV-3 antibodies were added. After an overnight incubation at 4 °C, 25 μl of a 50% slurry of protein A-Sepharose CL-4 B were added and incubation continued for 20 min at room temperature. After washing with lysis L buffer, bound proteins were eluted from protein A-Sepharose by boiling in 50 μl of 2 × concentrated dissociating buffer (0.05 M tris-Cl pH 6.8, SDS 2%, 2-β mercaptoethanol 2%, glycerol 30% and bromophenol blue 0.002%).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis—PAGE

Denatured proteins in dissociating buffer were separated by electrophoresis using a discontinuous buffer system [10]. Fluorograms were prepared as previously reported [3]. Molecular weights of proteins were calculated with respect to known standards electrophoresed on the same gel.

MHV-3 antibody

MHV-3 antibodies were produced in resistant A/J strain mice after two intraperitoneal injections of 10^3 PFU/mouse. Sera were collected 10 days after the second injection, pooled, and tested by immunofluorescence. For most experiments MHV-3 antisera were used at a final dilution of 1/100.

Results

Isolation and temperature sensitivity of ts mutants

Initial experiments showed that our wild type isolate of MHV-3 multiplied in L 929 cells within a temperature range commonly used for the isolation of animal virus mutants (33 °C to 39.5 °C). The rate of multiplication, however, depended on the incubation temperature. Maximum titers were reached 16 hours, 24 hours or 32 hours post infection for incubations at 33 °C, 37 °C, or 39.5 °C respectively. In order to induce mutation in the MHV-3 genome, the mutagens N-methyl-N'-nitrosoguanidine (NG) or 5-fluorouracil (FU) were used at concentrations that inhibited virus multiplication one hundred fold. Plaque isolates obtained from mutagenized stocks at 33 °C were then screened for their ability to produce syncytia at 39.5 °C. Potential mutants isolated using this criterion were further characterized by the efficiency of their plaque formation at 39.5 °C and 33 °C. Only mutants, whose relative plating efficiency 39.5 °C/

Table 1. Characterization of MHV-3 ts-mutants

Mutant	Titer at	Efficiency of plating[a]	Progeny yield PFU/ml			Plaque size at
	33 °C	39.5 °C/33 °C[b]	33 °C	37 °C/33 °C[b]	39.5 °C/33 °C[b]	39.5 °C
NG A 1312	2.8×10^5	1×10^{-2}	2×10^5	3×10^{-2}	5×10^{-4}	—
D 85	1.8×10^5	10^{-2}	4.4×10^5	1×10^{-2}	5×10^{-3}	small
D 222	1×10^5	$< 10^{-4}$	3.5×10^5	$< 10^{-4}$	$< 10^{-4}$	—
D 224	1.2×10^5	$< 10^{-4}$	3.3×10^5	1×10^{-2}	$< 10^{-4}$	—
D 265	4.6×10^4	$< 10^{-4}$	1.8×10^5	6×10^{-2}	$< 10^{-4}$	—
D 281	3.5×10^4	$< 10^{-4}$	1.5×10^5	8×10^{-3}	7×10^{-4}	small
D 282	2×10^4	$< 10^{-4}$	1×10^5	10^{-4}	$< 10^{-4}$	—
FU E 365	4×10^4	2×10^{-2}	3.2×10^5	2×10^{-2}	3×10^{-3}	small
F 72	4×10^4	$< 10^{-4}$	2×10^5	2×10^{-2}	$< 10^{-4}$	—
F 104	5.2×10^4	5×10^{-2}	1.6×10^5	7×10^{-2}	2×10^{-3}	—
F 111	4.2×10^5	3×10^{-2}	1.6×10^5	3×10^{-2}	6×10^{-2}	—
F 183	1.5×10^5	$< 10^{-4}$	2.2×10^5	$< 10^{-4}$	$< 10^{-4}$	—
Wt	6.1×10^5	0.5	7.5×10^5	9×10^{-1}	3×10^{-1}	—

[a] Ability to form syncytia at 39.5 °C. Ratio of the titer at 39.5 °C to the titer at 33 °C
[b] Ratio of the yields at 37 °C and 39.5 °C to the yield at 33 °C

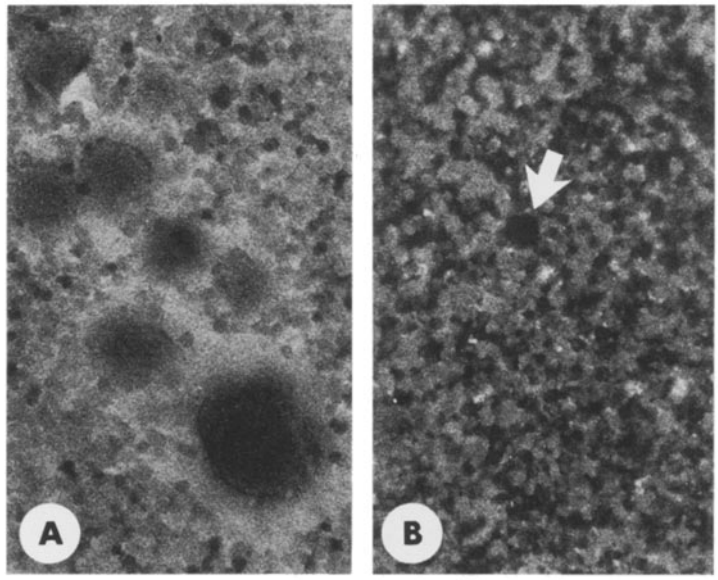

Fig. 1. Plaque size after 20 hr p.i. at 39.5 °C on L_2 cells after neutral red staining. Enlargement: × 660. **A** MHV-3. **B** Small plaque ts-mutant D 85

33 °C was lower than 5×10^{-2}, were kept for subsequent studies and a list of these mutants is given in Table 1. Under these conditions the mutation rate with NG was 1.2% and with FU 2.4%. Multiplication of the ts-mutants was studied at 33 °C, 39.5 °C, and also 37 °C as the last temperature is closest to the

Table 2. Yield of the progeny virus

	Percentage of the distribution			
	33 °C		39.5 °C	
Virus	Cell	Supernatant	Cell	Supernatant
Wt	44	66	37	63
D 85	40	60	88.2 (85)	11.7 (15)
E 365	58.5	41.5	91 (87)	9 (13)
E 20	86.6	13.3	98 (94)	2 (6)

Distribution of the virus yield in cells and supernatant at 33 °C and 39.5 °C, 20 hrs after infection
In parentheses, determination at 36 hrs after infection

body temperature of the mouse. Whilst several of the mutants appeared to be equally ts at 37 °C and at 39.5 °C, most of them were however less temperature sensitive at 37 °C. Several of the ts-mutants (D 85, D 281, E 365) produced small plaques at 39.5 °C (Fig. 1); in this case, the size of the plaques did not enlarge after 36 hrs. To understand this phenomenon, the yield in the medium and the yield remaining cell-associated were measured at 33 °C and 39.5 °C and compared with the yield of a non-ts small plaque mutant: E 20 (Table 2). The percentage of the yield obtained in the medium at 39.5 °C with mutants D 85 and E 365 was constant during infection (20 hrs and 36 hrs p.i.) and close to the percentage measured for mutant E 20. Small plaque formation therefore appeared to be related to the low release of virus in the external medium.

Complementation

The ability of mutants to complement each other was assayed by determining the yield of mixed infections at the nonpermissive temperature as compared to the yield of single infections. Complementation indexes (presented in Table 3) varied between < 1 and 95.2. When a CI greater than 3 was chosen as indicative of positive complementation, all mutants except one could be assigned to five different complementation groups. The inconsistent behaviour of mutant F 183 could possibly be explained if this mutant contained several mutations in different genes.

Viral RNA synthesis

The effect of different incubation temperatures on ^{3}H uridine incorporation into viral RNA was followed after incubation with the wild type or the ts

Table 3. Complementation of PFU production by double infection

	D 85	D 222	D 224	D 265	D 281	D 282	E 365	F 72	F 104	F 183	F 111
A 1312 83.3		93	26.6	22	30	18	16	53.3	*1.6*	3.3	*1.2*
D 85		10	4	9.5	*2*	9	*1.6*	9.5	23.3	20	19
D 222			*<1*	7.2	6.6	5.4	11.9	95.2	50	4	37
D 224				17.7	3.6	6.1	4.4	50	31.1	*<1*	26
D 265					6.6	4.9	14	*1.8*	16	*<1*	18.3
D 281						8.2	*1.8*	60	25	*<1*	10
D 282							12.6	22	17	7	11
D 365								16.2	8.5	4.7	17.5
F 72									16	*1.8*	20
F 104										6.2	*1.7*
F 183											14.2

Complementation groups

I	II	III	IV	V
D 222	D 265	D 282	D 85	A 1312
D 224	F 72		D 281	F 104
			E 365	F 111

mutants. In wild type infected cells, virus-induced RNA synthesis was maximal at 8 hrs, 10 hrs, and 12 hrs post infection at 39.5 °C, 37 °C, and 33 °C respectively. Mutants in groups I, II, and III failed to synthesize viral RNA at 39.5 °C and for these mutants synthesis was also reduced or abolished (group III) at 37 °C (Fig. 2A, B). Mutants in groups IV and V were able to induce RNA synthesis at a level similar to the wild type at 37 °C. At 39.5 °C uridine incorporation was halved with group IV mutants. Interestingly, one mutant F 104 (group V) displayed an accelerated rate of synthesis, particularly at the beginning of infection.

Negative-stranded RNA could not be detected by dot blot analysis of material from cells infected with RNA^- mutants (groups I, II, and II) at 39.5 °C (Fig. 2c). The group IV mutants showed a reduced synthesis of the negative RNA compared with the wild type virus at 6 hrs p.i.

The electrophoretic analysis of genomic RNA as well as of the subgenomic species of virus-specific intracellular RNAs for MHV-3 and mutants were similar (data not shown).

Virus-specific protein synthesis

The synthesis of MHV-3 proteins was followed either by immunofluorescence (IF) or immunoprecipitation (IP). At 33 °C the intensity of IF was similar in mutant and wild type infected cells. At 39.5 °C on the other hand, viral antigens

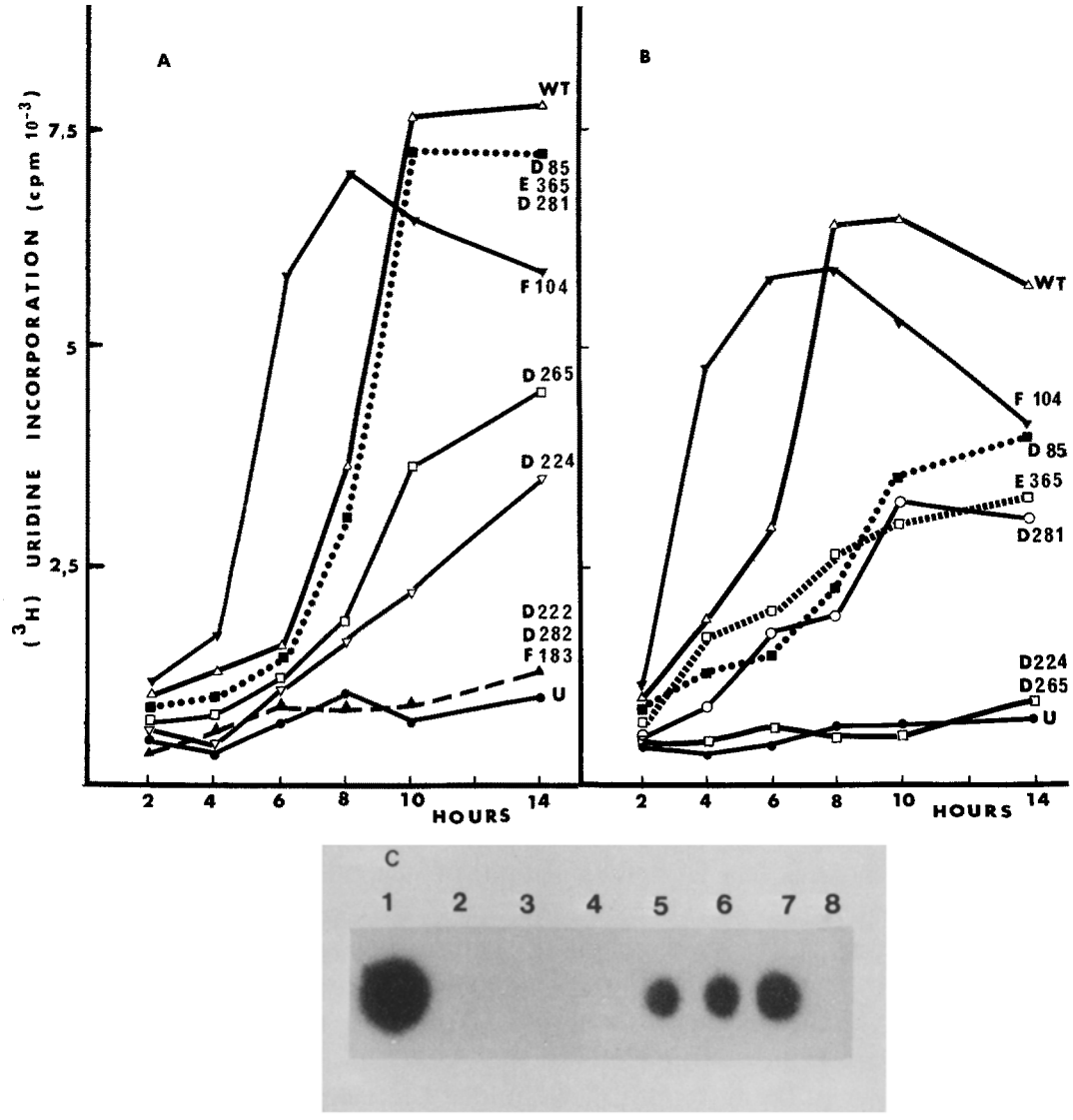

Fig. 2. Kinetics of viral RNA synthesis at different temperatures after infection of DBT cells with wild type virus and ts mutants (**A** at 37 °C, **B** at 39.5 °C). Viral RNA synthesis was measured by incorporation of ^{3}H uridine as described under Materials and methods. **C** Autoradiography of the RNA^{-} strand by dot blot analysis at 6 hrs p.i. The probe used was ^{32}P RNA obtained by transcription of the E 1 gene cloned in a T 7 transcription vector. *1* MHV-3, *2* D 222, *3* D 265, *4* D 282, *5* D 85, *6* E 365, *7* F 104, *8* mock-infected cells

could not be visualized in cells infected with mutants belonging to groups I or III and poor immunofluorescence appeared sparsely in a small number of cells infected with mutants D 265 and F 72. Ten mutants were tested for their ability to induce the synthesis of viral proteins by IP with polyclonal antibodies and subsequent polyacrylamide gel electrophoresis (Fig. 3). At 33 °C, cells infected with the wild type or various mutants displayed similar polypeptide patterns. The major species detected were E 2 (gp 180), N (pp 60), and, only weakly, E 1

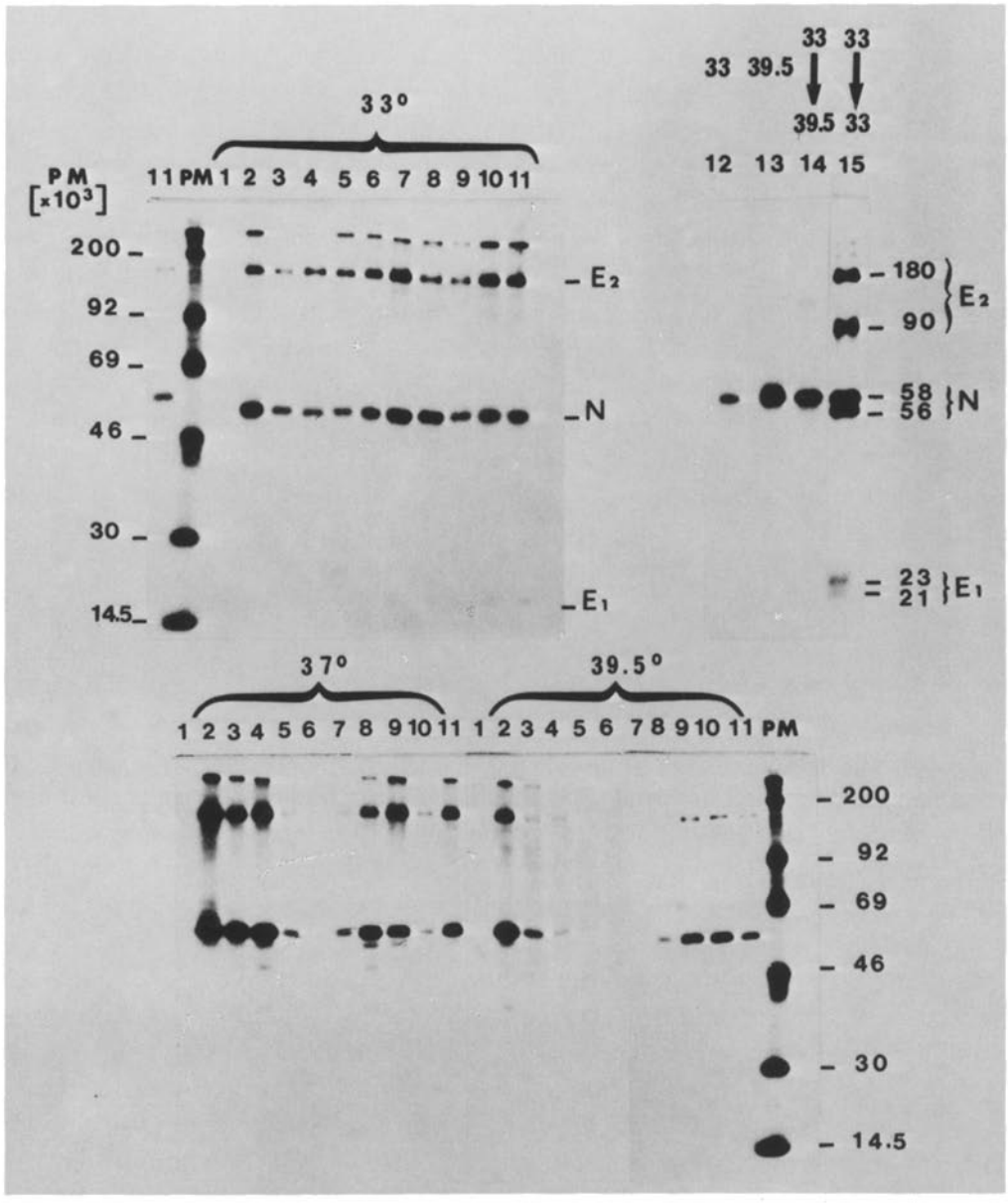

Fig. 3. Fluorogram of 12.5% polyacrylamide-SDS slab gel of the viral polypeptides labelled with ^{35}S methionine or ^{32}P orthophosphate and immunoprecipitated with MHV-3 polyclonal antibodies. *1–11* Viral polypeptides labelled for 30 min with ^{35}S methionine 10 hrs after infection at 33, 37, or 39.5 °C; the samples were collected immediately after labelling. *12–13* Viral phosphoprotein labelled for 2 hrs with ^{32}P orthophosphate at 10 hrs p.i. at 33 °C and 39.5 °C. *14–15*Pulse-chase of polypeptides or phosphoprotein labelled for 30 min with ^{35}S methionine (*15*) or for 2 hrs with ^{32}P orthophosphate (*14*) and collected 2 hrs later. *1* mock-infected cells, *2* MHV-3, *3* A 1312, *4* D 85, *5* D 222, *6* D 282, *7* D 265, *8* D 281, *9* E 365, *10* F 104, *11* F 111

(gp 21). The 90,000 mol wt protein, which is a product of post-translational processing of the 180,000 mol wt species, was barely detectable. Moreover, at 33 °C the apparent molecular weight of the N polypeptide was 2,000 smaller than its counterpart at 39.5 °C. However, after a pulse-chase labelling, two forms of the proteins N were detected. The molecule of 58,000 mol wt has the same electrophoresis mobility as the phosphorylated N protein product at 39.5 °C (Fig. 3). It could also be noticed that the phosphorylation was very efficient at 39.5 °C and poor at 33 °C; such a difference in the phosphorylation rate may explain the fact that the N pp is found after a short labelling at 33 °C as a molecule of 56,000 mol wt [26]. Mutants in groups I, II, and III failed to induce the synthesis of viral polypeptides at 39.5 °C and at 37 °C only mutants in group III did not synthesize proteins whereas the others displayed a reduction in the synthesis of viral proteins. The polypeptide species found with RNA-positive mutants from groups IV and V were identical to those in wild type infected cells.

Discussion

This paper reports the isolation and characterization of a series of MHV-3 ts-mutants. The isolation of ts-mutants with MHV-3 appeared to be less straightforward than with other MHV strains since the stock titer of virus was usually low and on account of severe virus inactivation at the non-permissive temperature, a plaque enlargement method could not be employed. Given the low rate of spontaneous mutation, we used the mutagens NG and FU. Among the 12 ts-mutants studied, 6 exhibited complete thermosensitivity at 39.5 °C and complete (D 222, D 282, F 183) or partial thermosensitivity at 37 °C (D 224, D 265, F 72). With 3 of the mutants, D 85, D 281, E 365, the progeny at 39.5 °C was considerably reduced and plaques obtained at the non-permissive temperature were usually smaller than those of the wild type. The small size of the plaques could be correlated with less efficient release of virus into the external medium.

On the basis of yields obtained from mixed infections at the non-permissive temperature the mutants could be divided into five complementation groups. The 5 most thermosensitive mutants belonged to 3 complementation groups, I, II, and III. Viral RNA synthesis was defective in cells infected by these mutants at 39.5 °C (RNA^- mutants). In group V the mutant F 104 began RNA synthesis earlier (at 5 hours) than the wild type virus (8 hours) and the maximum amount of progeny virus likewise appeared at an earlier time.

The RNA^+ groups complemented all three RNA^- groups. The complementation indexes were higher between the group V mutants and the four other groups, particularly A 1312 in which case the CIs were superior. The CIs between RNA^- mutants were generally low except for mutant F 72 (group II) and the group I mutants. But the CIs in general were low compared with the results obtained by Leibowitz et al. [17] for MHV JHM, a possible explanation for this being the difficulty of obtaining a high titer with MHV-3 on DBT cells or the high rate of thermoinactivation of this virus. It was not possible to arrange

mutant F 183 in a complementation group since it failed to complement more than one complementation group, thereby suggesting that F 183 has multiple ts lesions.

Our results with ts-mutants differ from those obtained with MHV JHM by Leibowitz et al. [17] who found five RNA^- complementation groups and one RNA^+; Van der Zeijst [28] described five complementation groups of MHV A 59, all RNA^-. The fact that most ts-mutants are RNA^- may be due to the large target size of the viral polymerase or to the involvement of several viral genes in RNA synthesis. The first event after infection is the translation of the 5′ end of the genome into one or more polymerase molecules corresponding to the early polymerase detected by Brayton et al. [4]. The second event is the synthesis of the leader sequence required to prime subsequent mRNA synthesis [1]. The small size of the leader (72 nucleotides) makes it an unlikely target for mutagenesis.

The detection of negative strand RNA with mutants from groups I, II, and III failed using RNA-RNA hybridization with either the genomic RNA or with E 1 probe. These experiments suggest that the early polymerase was defective at the non permissive temperature. Similar results were obtained with ts mutants of MHV A 59 [R. Baric, personal communication]. In contrast to previous reports we found two RNA^+ complementation groups but our selection procedure based on the reduction of syncytia formation at 39.5 °C and not on plaque enlargement could pick out some mutants not fully ts with a mutation in genes not needed for RNA synthesis. With mutants in group V, the efficiency of RNA synthesis was similar at 37 °C and 39.5 °C when compared with the wild type virus, but the progeny yield was reduced by one hundred. A mutation in a gene interfering with the viral maturation could explain these results; however, it is known that the mutant virions are more rapidly inactivated at 39.5 °C than the wild type virus and this may also account for the small yield of mutants. Biochemical evidence indicates that there are at least six MHV genes expressed late in infection which are probably not required for RNA synthesis.

The analysis of viral polypeptide synthesis by immunoprecipitation confirmed the thermosensitivity of the ts-mutants D 85, D 222, D 265, D 281 at 39.5 °C. The fact that, in the case of mutant D 265, viral antigens were revealed in a very small number of cells by immuofluorescence is not in contradiction with the other data as the amount of proteins produced must be extremely small and inferior to the level detectable by the immunofluorescence method. Surprisingly, some mutants of the group IV, although RNA^+, synthesized viral polypeptides only poorly at 39.5 °C.

No change in the electrophoretic mobility of viral polypeptides induced at 37 °C or 39.5 °C by MHV-3 and the ts-mutants was detected. However, the apparent molecular weight of the N polypeptide produced at 33 °C was different from those synthesized at 37 °C and 39.5 °C. This difference was due to the degree of phosphorylation of the polypeptide which was most efficient at higher

temperatures. There was no amplification of any single polypeptide induced by the ts mutants and this is similar to the results obtained for the other MHVs [2, 9, 20].

These studies provide the groundwork for further investigation into the pathogenic properties of MHV-3. It is not yet clear, however, as to whether the ts-lesions and the altered pathogenic effects of some of the mutants are due to the same mutations.

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