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Viral Hemorrhagic Septicemia of Rainbow Trout: Selection of a Thermoresistant Virus Variant and Comparison of Polypeptide Synthesis with the Wild-Type Virus Strain

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Serial passage of viral hemorrhagic septicemia virus at gradually increasing temperature selected for a variant virus that replicates at 25°C and has a low pathogenicity for rainbow trout. Viral hemorrhagic septicemia virus-specific polypeptide synthesis was examined in epithelioma papulosum cyprini cells infected with either a wild-type strain or a thermoresistant variant. The wild-type N and M_1 proteins were synthesized throughout the course of infection, whereas L, G, and M_2 were more actively translated later in the replication cycle. The wild-type strain was more cytotoxic at 25 than at 14°C despite the fact that no translation could be evidenced when the temperature was raised. When epithelioma papulosum cyprini cells were infected with the variant virus, the kinetic study was obstructed since protein synthesis was difficult to observe by the pulse method at a low multiplicity of infection and aborted when the multiplicity of infection was raised. The variant was less cytotoxic at 25°C than the wild-type virus.

The viral hemorrhagic septicemia (VHS) virus is a rhabdovirus, the protein structure of which resembles closely that of rabies virus (9, 11). The virus is of economical interest since it can infect rainbow trout (*Salmo gairdneri* Richardson) in fish farms where it is transmitted by contaminated water. The infected fish die from hemorrhages due to infection of the endothelium of blood capillaries, anemia due to lesions of the hematopoietic tissues, and impairment of the fluid balance due to the destruction of the excretory structures of the kidney. The clinical signs are consistent with cell killing by viral particles. VHS serotype 1 also replicates in several fish cell lines at an optimum growth temperature of 14°C (2).

Since with VHS virus both in vitro cultivation (3) and experimental trials on the animal (1) can be well controlled, VHS virus may be well suited to investigation of the relationship between structure and function. The first step is the isolation of mutants which would have lost at least part of their pathogenicity, and since the wild-type strain replicates at low temperatures, the aim was to select a thermoresistant, nonpathogenic variant.

This paper describes the selection of such a variant and compares its polypeptide profile with that of the wild-type strain.

MATERIALS AND METHODS

Cells and virus strains. The epithelioma papulosum cyprini (EPC) cell line originating from an epidermal hyperplasia of carp was used in this study because the cells grow over a larger range of temperatures (2) than rainbow trout gonad (RTG-2; 12) cells. EPC cells were serially cultured in Tris-hydrochloride-buffered Stoker medium supplemented with 10% tryptose and 10% fetal calf serum. Additional details about the monolayer cultures are published elsewhere (2). The F_1 Danish virus strain (3) was a gift from P. Vestergärd-Jorgensen and had been passed 100 times in tissue culture when we received it. The 07-71 strain had been passed five times since we isolated it from an infected fish. Both belong to serotype 1 (8).

Selection protocol. The variant virus selection protocol is described below and summarized in Table 1. Serial passages were done either in Corning flasks (75 cm^2) or in Nunc tubes (4 cm^2), and plaque titrations (3) were done in plastic petri dishes (8 cm^2).

Pathogenicity. The infectivity trials are described in detail elsewhere (1); briefly, 50 fish per duplicate aquarium were held immersed for 3 h in 3 liters of aerated water containing 5×10^4 PFU/ml, and then free-flowing water maintained at 8 to 12°C was turned on, and the experiment was continued for 4 weeks.

Preparation of ^{14}C-labeled virus. Corning flasks (150 cm^2) were seeded with 55×10^6 cells and maintained overnight at 30°C (2). Growth medium was then removed, and the cells were infected at an input multiplicity of infection (MOI) of 0.01 (wild type) or 0.02 (variant) PFU/cell. After adsorption for 1 h, 20 ml of labeling medium (1 volume of Stoker medium, 4 volumes of Earle saline, and 0.5 μCi of an amino acid mixture per ml; specific activity, 45 mCi/mg-atom of C; Commissariat à l'Energie Atomique, France) was added to each flask. The fluids were harvested after complete destruction of the monolayers (72 h at 14°C for the wild type; 24 h at 25°C for the variant) and

clarified by centrifugation. The supernatant was centrifuged for 3 h at 25,000 rpm in an SW 27 Beckman rotor through a cushion of 15% sucrose in TNE (0.15 M Tris-hydrochloride [pH 7.6], 0.15 M NaCl, 0.001 M EDTA). The virus was resuspended in TNE and frozen as 40-μl portions.

Cytoplasmic extracts. Either Nunc tubes seeded with 2.1×10^6 cells or Costar plates seeded with 10^6 cells per well were incubated for 5 h at 30°C so that the cells would firmly attach. The growth medium was then replaced by a prelabeling medium (diluted Stoker medium containing 0.3 μg of actinomycin D per ml). Cells to be labeled 15 to 20 h postinfection were immediately infected, whereas cells to be labeled during the beginning of the viral cycle were pretreated for 17 h with actinomycin D before infection. The volume of the inoculum was 0.1 ml. The resulting MOI and the incubation temperatures are given below. The virus was absorbed for 1 h at 14°C. At the indicated time postadsorption, the prelabeling medium was removed, and the cells were incubated for 1 h in labeling medium containing 2.5 μCi of ^{14}C per ml. The cells were then rapidly washed, resuspended in 0.1 ml of cold TNE, and frozen at −30°C until used. The protein content was determined after addition of sodium lauryl sulfate to a final concentration of 1%.

Sodium lauryl sulfate-polyacrylamide gel electrophoresis. Cell lysates and virus particles were mixed with 1 volume of 2X electrophoresis sample buffer (0.06 M Tris-hydrochloride [pH 6.7], 1% sodium lauryl sulfate, 10% glycerol, 2% 2-mercaptoethanol, 1 mg of bromophenol blue per ml), boiled for 1 min, and then fractionated by electrophoresis in discontinuous sodium lauryl sulfate-polyacrylamide slab gels, with Tris-glycine-sodium lauryl sulfate as electrode buffer (7) at a constant voltage of 75 V for the upper gel and 225 V for the lower gel. The gels were dried under vacuum and autoradiographed at room temperature on NS-2T Kodak no-screen film. The radiograms were scanned at 625 nm with a Gelman Dc D 16 scanner, which computed the absorbance of the bands.

RESULTS

Adaptation of the virus to thermoresistance. The F_1 strain of VHS virus had undergone 100 passages at 14°C in rainbow trout gonad cells (RTG-2; 12) but was immediately adapted to EPC cells at 14°C (Table 1; designated $F_{14\ (101)}$ [101 passages at 14°C with uncloned inoculum]) since the yield was similar to that of a recently isolated virus strain of the same serotype (Table 1; 07-71). When the cultures were maintained at 20°C, the virus yield was reduced by 80%, and no virus could be recovered at 23°C (Table 1) (3).

The culture medium from cells infected with the F_1 isolate (Table 1; $F_{14\ (101)}$) was diluted 10^{-2} and used as an inoculum for passage 1 at 20°C. On serial passage 4 at that temperature, complete destruction of the monolayer was observed with an inoculum diluted 10^{-3}, suggesting that adapted virus particules were probably already

TABLE 1. *Adaptation of the virus to thermoresistance*[a]

Temp (°C)	No. of following virus recovered:										
	07-71[b]	$F_{14\ (101)}$[c]	$F_{20\ (20)}$	$F_{20\ (45)}$[d] ↑↑↑	$C_{20\ (46)}$	$F_{23\ (4)}$ ↑↑↑	$C_{23\ (5)}$	$F_{23\ (7)}$	$F_{23\ (14)}$..... ↑↑↑	$F_{25\ (3)}$	$F_{25\ (21)}$[e]
14	3×10^8	2.6×10^8[f]	4×10^8	6×10^8	4×10^8	6×10^6	5×10^6 3×10^6 5×10^6	1.2×10^8[f]	9.3×10^7[f]	ND[g]	6×10^8
20	—[h]	—[h]	3×10^8	6×10^8	3.4×10^8	ND	ND	ND	ND	ND	ND
23	0	0	ND	ND	1.2×10^8	9×10^6	7.5×10^6 5×10^6 5×10^6	8.3×10^7	6×10^7	ND	ND
25					0	ND	ND	ND	ND	1.4×10^5	4×10^8

[a] All titrations were done at 14°C. Arrows indicate that the virus has been cloned.
[b, c, d, e] Pathogenicities are as shown in Fig. 2a, b, c, and d, respectively.
[f] Average of three independent determinations.
[g] ND, Not done.
[h] —, See text.

efficiently selected. After 20 blind passages, the F_{20} variant was as efficient at 20°C as the F_1 strain was at 14°C, and further passages did not improve the adaptation at 20°C or impair the ability to multiply at 14°C (Table 1; $F_{20\ (20)}$, $F_{20\ (45)}$).

After 45 blind passages at 20°C, a clone (C) was selected and passed once more ($C_{20\ (46)}$), and then the yield at all four temperatures was titrated. No virus could be recovered at 25°C, but the virus could replicate at 23°C as efficiently as at 14°C. In an effort to verify if the efficiency to replicate in that range of temperature was a property of the individual virus particles or due to a heterogeneity of the population, the virus was then cloned repeatedly, first after passage 4 at 23°C ($C_{23\ (5)}$; three different clones) and then successively six times after passage 14 ($C_{23\ (15\rightarrow20)}$; data not shown). All clones retained the ability to multiply on the full temperature range. On the other hand, after passage 7 at 23°C (Table 1; $F_{23\ (7)}$) the virus was already fully adapted since the new variant was as efficient at 23°C as the wild-type was at 14°C. Further passages did not improve the yield.

Passage 22 at 23°C was used as an inoculum for passage 1 at 25°C. Selection of virus that would replicate at that temperature occurred by passage 3 (Table 1; $F_{25\ (3)}$).

One-step growth kinetics of virus replication with the uncloned $F_{25\ (21)}$ variant and 07-71 wild-type isolate at 10, 14, and 25°C are shown in Fig. 1. No virus could be recovered from the wild-type sample held at 25°C. When the desorbed virus (2 h) was substracted from each point, the yield was the same for the variant at each of the three temperatures as for the wild type at 10 and 14°C, but the virus replication cycle was shortened when the temperature was raised, since with the thermoresistant virus the maximum yield was obtained after 15 h at 14°C and 5 h at 25°C, so that 25°C can be considered as the optimum temperature for this variant.

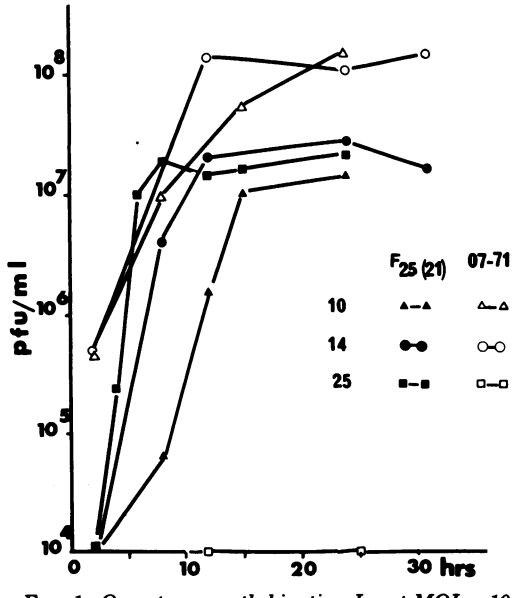

FIG. 1. *One-step growth kinetics. Input MOI = 10 PFU/cell. Titration was done at the same temperature as that for the multiplication cycle.*

Thus, it has been possible to select a variant of VHS_1 which was able to replicate at 25°C, and this result was obtained by several successive mutational events. Once a variant was selected, the progeny would rapidly multiply at the new permissive temperature, and in about five to seven passages, the yield would be sufficient so that a new mutation had a good probability of occurring.

Pathogenicity. At different times during the adaptation process, the pathogenicity of the virus for the rainbow trout was tested. The F_1 isolate retained its pathogenicity after 101 passages at 14°C (Fig. 2b) plus 45 passages at 20°C (Fig. 2c) as compared with the 07-71 recent isolate (Fig. 2a) since, respectively, 95, 84, and 98% of the infected fish died. Most of the fish were dead within 7 days postinfection. Thus, a total of 146 subcultures in vitro were not sufficient to attenuate the virus.

No clear-cut differences in the pathogenicity of the F_{23} variant could be detected because the results varied from one experiment to another (data not shown). This could be due to some instability of the pathogenic properties during the adaptation to thermoresistance. In contrast, most of the fish which were exposed to the $F_{25\ (21)}$ variant (Fig. 2d) survived, and the 4.5% that finally died did so between 9 and 17 days postinfection. In another experiment (data not shown), we verified that virus could be recovered from at least 50% of the survivors assayed by using EPC monolayers and that 84% were protected against a challenge with the 07-71 wild-type strain, which killed 89% of the control. Thus, most of the fish had been infected, but the virus was attenuated. The critical mutational event probably happened during adaptation to 23°C. The new phenotype was then preferentially selected at 25°C.

Viral proteins. The polypeptides of the wild-type 07-71 virus grown at 14°C could be resolved into the five polypeptide species which have already been described (9, 11), but in all our gels, a small peak was observed slightly ahead of the N protein (Fig. 3; N_x). This peak has not been described yet, probably due to the fact that other authors used cylindrical gels. Whether or not this protein is the equivalent of the NS

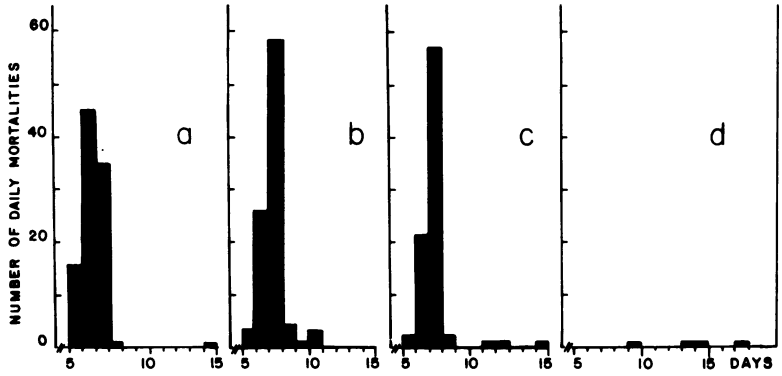

FIG. 2. *Daily mortalities obtained in infection trials with different VHS$_1$ virus strains. (a) Wild-type strain 07-71; (b) $F_{14\ (101)}$; (c) $F_{20\ (45)}$ variant; (d) $F_{25\ (21)}$ variant. For these trials, duplicate groups of 50 fish each (mean weight, 1 to 2 g) were immersed for 3 h in 3 liters of contaminated water (5 × 10^4 PFU/ml) and were subsequently held for 4 weeks in aquariums supplied with tap water at 10 to 11°C.*

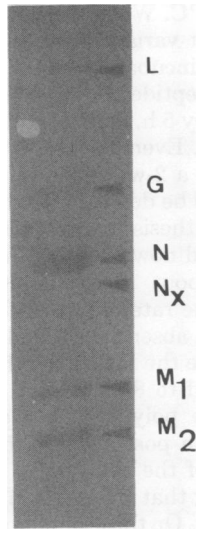

FIG. 3. *07-71 viral proteins. EPC cells infected with the wild-type strain at an input MOI of 0.01 PFU/cell were maintained in labeling medium (no actinomycin D) for 72 h at 14°C. After destruction of the monolayers, the virus was harvested, concentrated, and purified as described in the text.*

protein of vesiculoviruses is unknown. The migration of the five major polypeptides was strictly proportional to the $\log_{10}$ of the published molecular weights (9). Thus, the relative number of molecules for each polypeptide could be calculated based on the average of the densitometer tracings from five autoradiograms (Table 2). Assuming one N molecule (chosen for standardization), the ratios were 0.05 L, 0.16 G, 0.6 M_1, and 1.3 M_2.

Since the trials on fish had been done with $F_{25\ (21)}$ grown at 25°C, the same temperature was chosen to study the viral polypeptides of that variant; only three species, N, M_1, and M_2, were resolved. We verified that the sensitivity of the scanning was sufficient for L and G to be seen if they had been represented in the same percentage as for the wild-type virus (Table 2).

Since VHS virus multiplication did not inhibit cellular protein synthesis (data not shown), the cells had to be pretreated with actinomycin D so that virus-specific polypeptides could be recognized in cytoplasmic extracts. This method was preferred to hypertonic medium, which has been shown to modify the rate of synthesis of rabies G protein (10). The relative incorporation of the radioactive precursors in the virus-specific polypeptides was unchanged whether the prelabeling medium was added after adsorption of the virus or the cells were pretreated (data not shown). All mock-infected cells were still firmly attached after 24 h of treatment with actinomycin D whichever the incubation temperature was.

Wild-type 07-71 at 14°C. At an input multiplicity of infection of 20 PFU/cell, the elecrophoretic profile of the cytoplasmic extracts from cells pulse-labeled at 1 h postinfection was the same as that of the mock-infected cells (Fig. 4). The synthesis of the polypeptides N and M_1 began 2 h, M_2 and G began 8 h, and L began 16 h postinfection (arrowheads on Fig. 4). Since at 8 h the rate of synthesis increased as a function of time, as shown by the increasing height of the peaks obtained during each 1-h pulse (also visible on Fig. 4), this amplification may mean that secondary transcription on replicated viral genomes had occurred. By 20 h postinfection, the cells began to round up, and by 24 h, most of them had detached.

At an input MOI of 14 PFU/cell, the cells detached later (24 to 30 h).

Wild-type 07-71 at 25°C. In contrast, when

TABLE 2. *Characteristics of viral proteins*[a]

Protein	Mol wt	07-71 (14°C)[b]		$F_{25\,(21)}$ (25°C)[c]	
		% OD	Relative no. of molecules	OD observed	OD expected
L	190,000	9.7	0.05	0	908
G	80,000	12.4	0.16	0	1,161
N	38,000	37.7	1	3,559	3,529
M_1	25,000	14.6	0.6	3,337	1,366
M_2	19,000	25.6	1.3	2,467	2,396

[a] Labeled viral particles were fractionated by electrophoresis as described in the text and the gels were autoradiographed.

[b] By using the relative optical density (OD) of each band in the autoradiogram, the relative number of polypeptide molecules was determined, assuming the N protein equals one molecule.

[c] By using the total OD (9,363 arbitrary units), the expected OD was calulated by assuming that the percentage would be the same as that for 07-71.

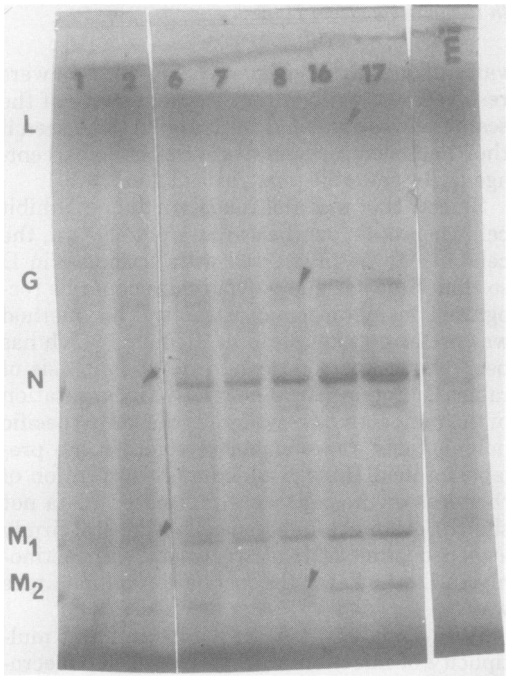

FIG. 4. *Cytoplasmic extracts, 07-71, at 14°C. The MOI was 20 PFU/cell. Numbers at the top of each well indicate the end of the 1-h pulse. mi, Mock-infected cells treated with actinomycin D for 24 h. Arrowheads indicate beginning of the synthesis.*

cells infected at an input MOI of 14 PFU/cell and maintained at 14°C during adsorption to avoid inactivation of the inoculum were transferred to 25°C, they rounded up and detached by 5 to 6 h postadsorption, and no incorporation of the radioactive precursors could be seen on the autoradiograms (data not shown), even with a protein content of 200 μg/well and 2 weeks of exposure on the Kodak film. Thus, the wild-type virus inoculum was more cytotoxic at 25 than at 14°C despite the fact that the virus genome could not be efficiently translated when the temperature was raised.

$F_{25\,(21)}$ at 14°C. When cells infected with the thermoresistant variant at an input MOI of 20 PFU/cell were incubated at 14°C (Fig. 5), traces of the N polypeptide could be seen by 1 h, M_1 could be seen by 5 h, and M_2 could be seen by 7 h postinfection. Even with a protein content of 75 μg/well and a 2-week exposure on the film, no G or L could be detected. On the other hand, the rate of synthesis increased from 1 to 16 h and then slowed down after 17 h, and the cells detached by about 30 h postinfection. At no moment was the rate of synthesis high enough to ascertain the absence of L and G.

To ameliorate the rate of synthesis, the input MOI was raised to 80 PFU/cell (0.2-ml inoculum). All three polypeptides were visible between 2 and 7 h postinfection, but the rate of incorporation of the radioactive precursor was constant during that time (Fig. 6), and L and G were still absent. On the other hand, all synthesis was abruptly turned off by 8 h, but the cells detached by only 24 h postinfection.

$F_{25\,(21)}$ at 25°C. When cells infected with the thermoresistant variant at an input MOI of 2 PFU/cell (diluted inoculum) were maintained at 25°C, the cells detached by 24 h postinfection, but the level of synthesis was too low, and the polypeptides could not be labeled by the pulse method.

At an input MOI of 40 PFU/cell, the cells were still attached by 8 h postinfection, and thus, the cytotoxic effect at 25°C was reduced as compared with the wild type at the same temperature. All cells were detached by 16 h postinfection. On the autoradiograms (Fig. 7), traces of N were visible between 3 and 7 h, but the bands were so faint that the absence of the other polypeptides was not significant.

A summary of the results is presented in Table 3.

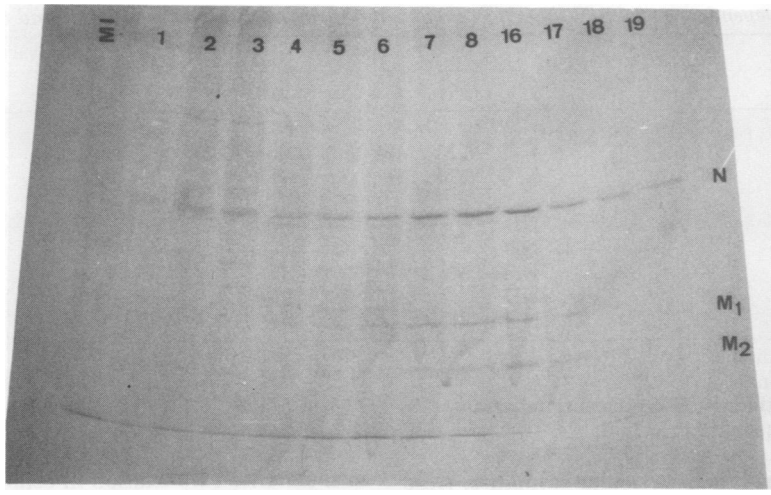

FIG. 5. *Cytoplasmic extracts, $F_{25\ (21)}$, at 14°C. The MOI was 20 PFU/cell. MI, Mock-infected cells treated with actinomycin D for 24 h. Numbers at the top of each well indicate the end of the 1-h pulse.*

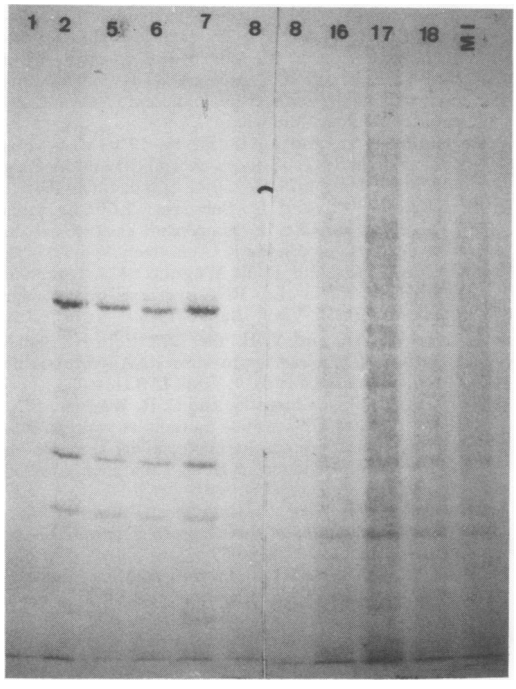

FIG. 6. *Cytoplasmic extracts, $F_{25\ (21)}$, at 14°C. The MOI was 80 PFU/cell (0.2 ml). MI, Mock-infected cells treated with actinomycin D for 24 h. Numbers at the top of each well indicate the end of the 1-h pulse.*

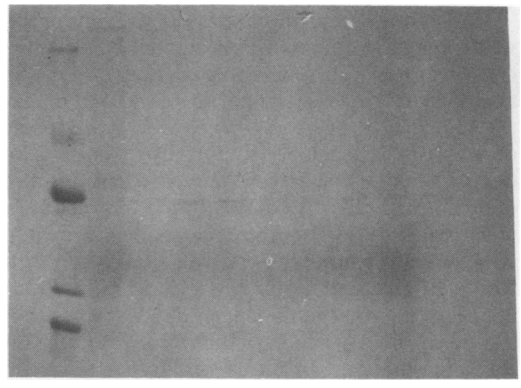

FIG. 7. *Cytoplasmic extracts, $F_{25\ (21)}$, at 25°C. The MOI was 40 PFU/cell. First well, 07-71 viral proteins as migration markers.*

DISCUSSION

A variant of VHS virus has been selected which can multiply in EPC cells over the range 10 to 25°C. After each step of the adaptation process, the new variants were as efficient for inducing in vitro cell lysis at the new permissive temperature and low input MOI as they were at 14°C, and the yields obtained at both temperatures were similar when compared by plaque titration at 14°C (Table 1). Thus, in the range of temperatures to which they are adapted, the variants could carry out all the functions leading to cell lysis and the release of viral progeny. During the adaptation process to thermoresistance, the pathogenicity of the virus was attenuated since most of the fish exposed to the virus survived, and the few fish which died did so significantly later than the control (Fig. 2). Thus, the infecting viral particles were unable to kill the fish despite the fact that they could still lyse the cells in vitro. This may be related to the fact that the viral particles of the $F_{25\ (21)}$ inoculum lacked normal amounts of L if that polypeptide is as necessary for virus-specific RNA transcrip-

TABLE 3. *Dependence of cell-killing effect and viral protein synthesis on input MOI and temperature*

Virus	MOI	Proteins		Survival time (h)	
		14°C	25°C	14°C	25°C
07-71	0.01[a]	ND[b]	ND	72	Survived
	14	L, G, N, M_1, M_2	None[c]	24–30	5–6
	20	L, G, N, M_1, M_2	ND	18–24	ND
$F_{25\ (21)}$	0.02[a]	ND	ND	72	24
	2[a]	ND	None	ND	<16
	20	N, M_1, M_2	ND	24–30	ND
	40	ND	None	ND	<16
	80[d]	N, M_1, M_2	ND	24	ND

[a] Diluted medium.
[b] ND, Not done.
[c] No polypeptide visible on the autoradiogram.
[d] A 0.2-ml amount of inoculum.

tase activity as it is in vesicular stomatitis virus (4, 5, 6). Thus, the replication of the virus may be slowed down sufficiently so that the defense mechanisms of the fish could overcome the infection.

In EPC cells and in parallel experiments, the wild-type inoculum at an input MOI of 14 PFU/cell was more cytotoxic at 25 than at 14°C. The necessity of a rise in temperature indicated that the cell killing effect in that case was the result of an enzymatic reaction, but the lysis may be due either to a nonspecific factor released in the undiluted culture medium which served as a virus stock or to the abortive replication cycle when cells infected at 14°C were transferred to 25°C. In the first hypothesis, it is difficult to understand why the nonspecific factor was lacking in the $F_{25\ (21)}$ stock, which was prepared in the same manner. In the second hypothesis, the critical event happened before translation, since no polypeptide synthesis could be observed.

The kinetic study of virus-specific polypeptides in cells infected with the thermoresistant variant was obstructed since protein synthesis was difficult to observe at a low MOI and aborted after 8 h when the MOI was raised. This result cannot be explained by interferon production since the cells were maintained under actinomycin D; on the other hand, exogenous interferon is usually efficient only if the cells are pretreated before adsorption of the virus.

One possible hypothesis is that at a high MOI the variant disturbs the host cell, and secondary transcription or translation or both becomes impossible; it may be significant that at 14°C the shutoff of protein synthesis in cells infected with the thermoresistant variant was contemporary with enhanced translation in cells infected with the wild-type virus.

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