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Difference in Sensitivity to Interferon among Mouse Hepatitis Viruses with High and Low Virulence for Mice

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Mouse hepatitis viruses (MHV) of different virulence for mice were studied with respect to interferon (IFN) sensitivity. The growth of low-virulent MHV-S and intermediately virulent MHV-JHM was significantly suppressed in IFN-treated L cells compared with untreated cells. However, a comparable suppression of the growth of highly virulent MHV-2 was not observed in IFN-treated cells. This differential effect of IFN treatment could also be demonstrated at the level of viral mRNA and viral proteins. In cells infected with MHV-S or MHV-JHM the amount of viral mRNAs was remarkably reduced by IFN treatment. Also the levels of the major intracellular viral proteins, in particular the E_1 protein, were affected by IFN treatment. Similar effects could not be demonstrated in MHV-2-infected cells. These results suggest that during MHV-S or MHV-JHM infection IFN treatment suppresses virus replication at several stages. The significance of these results is discussed in terms of the pathogenecity of these viruses.

INTRODUCTION

Mouse hepatitis virus (MHV) is a member of the coronavirus family which are enveloped viruses with large positive stranded RNA genomes (Siddell *et al.*, 1983, Sturman and Holmes 1983). In MHV-infected cells, seven mRNAs, which form a 3′ coterminal nested set, are synthesized (Spaan *et al.*, 1983). These mRNAs are thought to be transcribed from a single species of negative-stranded RNA, which has the same size as genomic RNA (Lai *et al.*, 1982). MHV virions contain, in addition to the genome RNA, a nucleocapsid protein (NP), a glycosylated membrane protein (E1), and a glycosylated surface protein (E2). The polypeptide components of these proteins are translated from mRNAs 7, 6, and 3, respectively (Rottier *et al.*, 1981; Siddell *et al.*, 1980).

The pathogenesis of MHV infections has been widely investigated, since they provide good animal models for disease in humans and domestic animals (Piazza, 1969). MHV infections are also ideal experimental systems to investigate mechanisms of resistance to viral infection (Wege *et al.*, 1982). Originally Bang and Warwick (1960) were able to demonstrate that the resistance of specific mouse strain to MHV-2 infection could be accounted for by the genetically determined resistance of macrophages. This phenomenon has subsequently been reported not only for a variety of MHV infections (Stohlman *et al.*, 1981; Taguchi *et al.*, 1976; Virelezier and Allison, 1976) but also for other viral infections (Mogensen, 1979). However, it is now also believed that IFN is also involved in the resistant mechanisms during MHV infection (Taguchi *et al.*, 1979; Virelezier *et al.*, 1977; Virelezier and Gresser, 1978). For example, it is known that IFN can render susceptible macrophages resistant to MHV infection in culture (Virelezier *et al.*, 1977), and also that anti-IFN serum makes mice more susceptible to acute and chronic diseases caused by MHV (Virelezier and Gresser, 1978). However, the mechanisms of IFN-mediated resistance at the cellular level have not been studied. We report here the difference in sensitivity to IFN among MHV strains with different virulence for mice and the effect of IFN on the multiplication of these viruses.

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MATERIALS AND METHODS

Cells. DBT cells (Kumanishi, 1967) and L cells were used for the titration of infectious viruses as described previously (Taguchi *et al.*, 1976). L cells were used to determine the effects of IFN on MHV replication. DBT and L cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 10 and 5% fetal calf serum (FCS), respectively.

Virus and virus assay. Three strains of MHV with different virulence for mice (Hirano *et al.*, 1981; Taguchi *et al.*, 1981); MHV-2 (high), JHM (intermediate), and MHV-S (low), were used. These viruses were propagated in DBT cells as described previously (Taguchi *et al.*, 1976).

IFN. Mouse alpha interferon (1×10^5 u/ml) was purchased from Lee, Biomolecular Research Inc., San Diego, California, and the titration of IFN was performed using L cells and vesicular stomatitis viruses (Taguchi *et al.*, 1976), both provided by Dr. C. Jungwirth.

RNA extraction. L cells cultured in 3-cm petri dish were infected with MHV-2, MHV-S, or MHV-JHM at a multiplicity of 1 PFU/cell at 37° for 1 hr. The cells were then washed once with EMEM and incubated with EMEM-supplemented with 2% FCS. Twelve hours p.i., the cells were collected by scraping with a rubber policeman, lysed with phosphate-buffered saline, pH 7.2 (PBS), containing 0.65% NP40, and RNA was isolated by phenol extraction as described previously (Siddell, 1983; Taguchi *et al.*, 1985).

Northern blot analysis. Northern blot hybridization was performed as previously described (Maniatis *et al.*, 1982; Taguchi *et al.*, 1985). Briefly, RNA extracted from cells was electrophoresed in agarose gels containing formaldehyde using MOPS buffer. After electrophoresis, the gel was sequentially treated with 50 m*M* NaOH, 100 m*M* Tris-HCl, pH 7.6, and 20 × SSC (1 × SSC is 0.15 *M* NaCl, 0.015 *M* sodium acetate, pH 7.0). RNA was transferred onto nitrocellulose paper and immobilized by baking at 80° *in vacuo.* The filter was hybridized with cDNA complementary to mRNA 7 of JHM (Skinner and Siddell, 1983) which had been labeled with ^{32}P by nick translation. Prehybridization and hybridization were performed as described previously (Taguchi *et al.*, 1985). The filter was washed at 50° with 2 × SSC containing 0.1% sodium dodecyl sulfate (SDS) and 0.1 × SSC containing 0.1% SDS before exposure to X-ray film at −70°.

Labeling and immunoprecipitation of intracellular viral proteins. L cells were infected with virus as described above. Eleven hours p.i., the culture medium was replaced with EMEM without methionine and cells were cultured for 1 hr. Thirty minutes before cell harvest, [^{35}S]methionine (SJ204, Amersham, Braunschweig, FRG) was added to the medium at a concentration of 30–50 μCi/ml. Cell lysates were prepared with PBS containing 0.5% NP40 as described previously (Taguchi *et al.*, 1985). Immune precipitation using anti-JHM rabbit serum, as well as SDS-polyacrylamide electrophoresis were performed as described previously (Siddell *et al.*, 1981).

Cell-free protein synthesis. A micrococcal-nuclease treated rabbit reticulocyte lysate prepared as reported by Pelham and Jackson (1976) was used for *in vitro* translation. Poly(A) containing RNA was selected by poly(U)-Sepharose (Siddell, 1983). The *in vitro* translation mixture contained 6 m*M* 2-amino-purine, 200 μg/ml of tRNA, and 20 μCi of [^{35}S]methionine in 10 μl reticulocyte lysate, to which 2 μl of poly(A)$^+$ RNA was added. The mixture was incubated at 32° for 1 hr as previously described (Siddell *et al.*, 1980). Aliquots of the samples were precipitated by anti-JHM serum and precipitates were electrophoresed on SDS-polyacrylamide gel.

RESULTS

Sensitivities of MHV Strains to IFN

L-cell monolayers were treated with 10-fold dilutions of mouse alpha IFN for 12 hr, and were then infected with MHV-2, MHV-S, or MHV-JHM at a multiplicity of 0.1 or 0.01 PFU per cell. The infectivities of these challenge viruses were determined by plaque assay on L cells, and not DBT cells, because the plaque efficiency on L cells and DBT cells differs, depending on

the strain of MHV. The infectivities in the culture fluid collected at 24 hr (Fig. 1a) and 12 hr (Fig. 1b) p.i. were determined by plaque assay on DBT cells. As shown in Fig. 1, MHV-S and MHV-JHM were highly sensitive to IFN treatment, and the effect is greater when cells were infected with lower multiplicities. In contrast, MHV-2 showed considerably higher resistance to IFN treatment. The cytopathic effects (CPE) caused by MHV-S and MHV-JHM on L cells were suppressed by IFN treatment, and the degree of suppression correlated with the concentration of IFN used (data not shown). MHV-2 did not produce polykaryocytes in infected cells.

Effect of IFN on the Synthesis of Viral mRNA

As a prelude to studying the effect of IFN on viral mRNA, we first of all examined the effect of IFN treatment on virus growth at a multiplicity of infection (1 PFU per cell) necessary for biochemical studies. After 12 hr pretreatment of the cells with a concentration of 1000 u/ml IFN the growth of MHV-S and MHV-JHM was approximately 95% reduced, while MHV-2 growth was reduced by only 40 to 50%. Under these conditions, we examined the amount of viral mRNAs in IFN-treated and -nontreated cells by Northern blot hybridization. As shown in Fig. 2a, the levels of all species of viral mRNA were suppressed in IFN treated cells infected with MHV-S or MHV-JHM as compared with untreated cells. Densinometric analysis of autoradiograms showed that the reduction of mRNA 2 and 3 is greater (more than 95% reduction) than that of mRNA 6 and 7 (about 76% reduction) (Fig. 2b). On the contrary, in MHV-2 infection, there is no such remarkable difference in the amount of mRNAs between IFN-treated and -untreated cells. Comparison by densinometric analysis of the autoradiograms showed 58, 34, and 5% reduction in mRNA 3, 6, and 7, respectively. Quantitative analysis of mRNA 1 was difficult, since only trace amounts of mRNA 1 were detected even in IFN-untreated cells, although a difference in the amount of mRNA 1 in IFN-treated and -untreated cells is apparent in Fig. 2a.

Effect of IFN on Viral Proteins

Virus-specific proteins in infected cells were studied by immune precipitation and SDS-polyacrylamide gel electrophoresis. Conditions for IFN treatment and infection were the same as the experiment described above. As shown in Fig. 3, the levels of MHV-S and MHV-JHM proteins were reduced in cells treated with IFN as compared with untreated cells, whereas the amount of MHV-2-specific proteins was not significantly affected by IFN treatment. Furthermore, in the case of MHV-S and MHV-JHM densinometric analysis of the

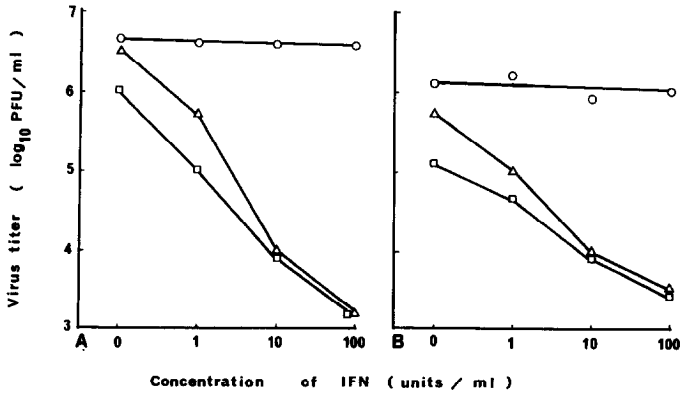
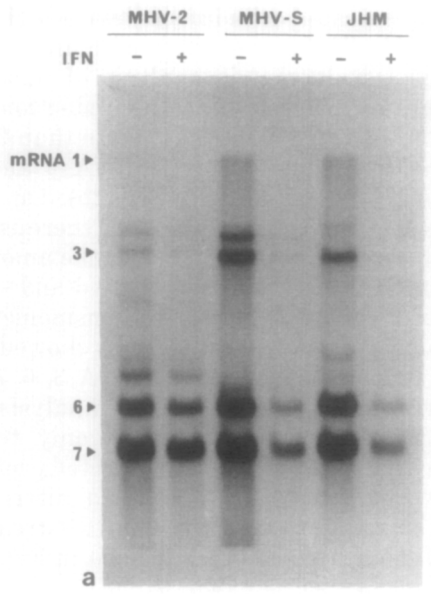
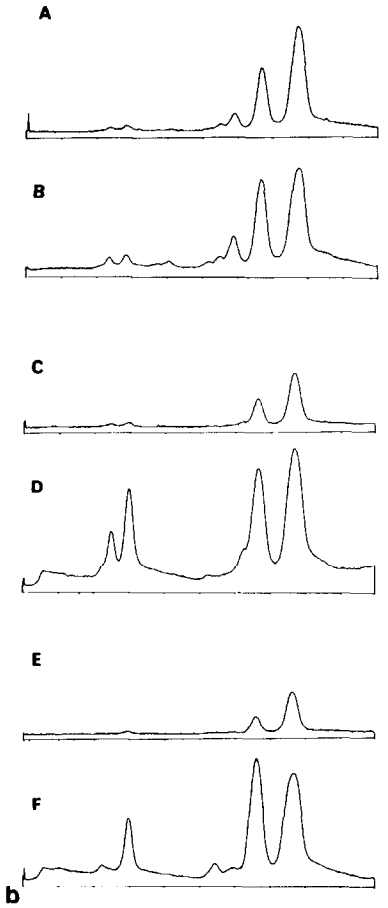


FIG. 1. L cells pretreated for 12 hr with various concentrations of IFN were infected with three strains of MHV and virus titers in the supernatants were plaque assayed at the time indicated in text. MHV-2 (○), MHV-S (△), MHV-JHM (□). (A) m.o.i. = 0.01. (B) m.o.i. = 0.1.





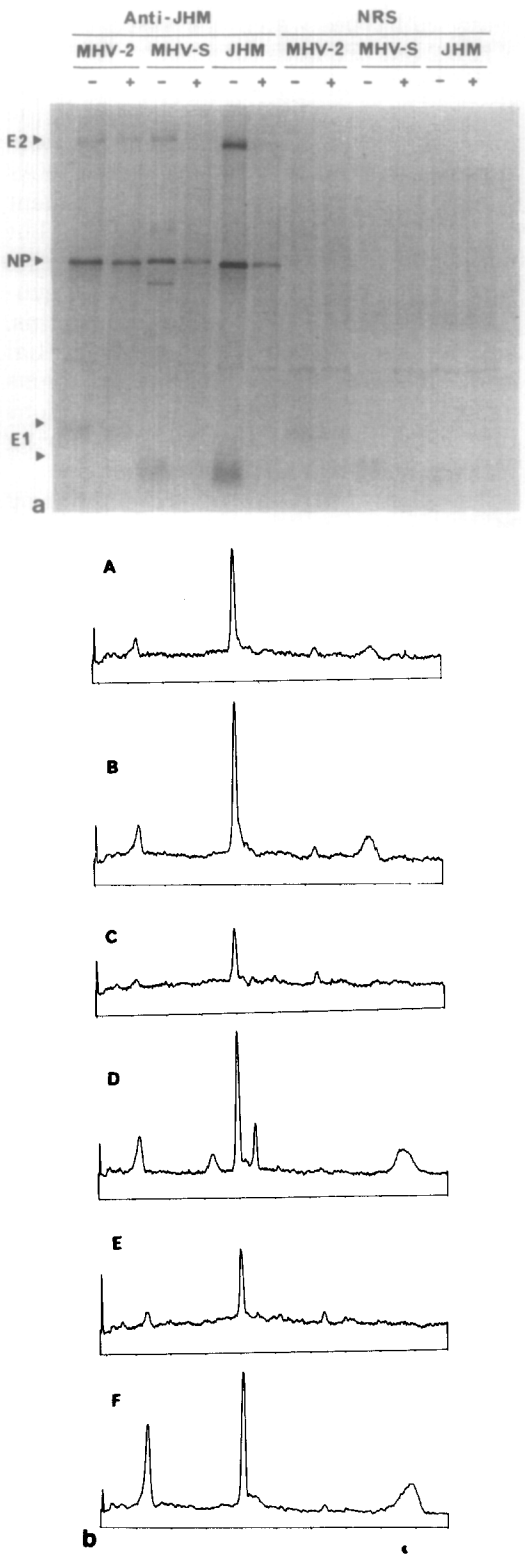
autoradiograms revealed that IFN treatment had a differential effect on the major viral proteins. The E1 proteins were most drastically affected (more than 95% reduction, Fig. 3b) whereas the nucleocapsid proteins (30–50% reduction) and the E2 proteins (70–80% reduction) were less affected.

This differential effect on the E1 protein was more vividly demonstrated by a reconstruction experiment in which diluted lysates from infected untreated cells were immunoprecipitated in parallel with the undiluted lysate from infected IFN-treated cells. Figure 4 shows that in the case of MHV-S and MHV-JHM but not MHV-2, proportionally less E1 protein is present in the lysate from IFN-treated cells than in diluted lysate from untreated cells which contained approximately comparable amounts of N or E2 protein. This demonstrates that the synthesis or stability of the E1 protein is preferentially affected by IFN treatment.

In Vitro Translation of mRNA from MHV-S-Infected Cells Treated with IFN

We have shown that IFN treatment leads to a reduction in the levels of viral mRNAs and proteins during MHV-S and MHV-JHM infection and that the effect is disproportionally large on the level of intracellular E1 protein, although the amount of mRNA 6, which encodes this protein, does not appear to be specifically depressed. To test whether mRNA 6 remains functional after IFN treatment we examined the capacity of mRNA 6 from MHV-S-in-

FIG. 2. (a) Comparison of mRNA by Northern blot analysis. L cells pretreated (+) or untreated (−) with IFN for 12 hr were infected with MHV-2, MHV-S, or MHV-JHM at a m.o.i. 1 and RNA was extracted at 12 hr p.i. RNA (5 μg) was electrophoresed, transferred to nitrocellulose paper, and hybridized with ^{32}P-cDNA complementary to mRNA 7 of JHM. (b) Densinometric analysis of autoradiogram shown in Fig. 2a. A: MHV-2, IFN (+). B: MHV-2, IFN (−). C: MHV-S, IFN (+). D: MHV-S, IFN (−). E: MHV-JHM, IFN (+). F: MHV-JHM IFN (−).



fected cells, with or without IFN treatment, to translate the E1 protein precursor *in vitro*. Prior to translation the amount of mRNA 6 in each sample was determined by Northern blot analysis (Fig. 5b) and Fig. 5a shows that allowing for the slightly lower amounts of mRNA 6 in the sample from the IFN-treated cells, the *in vitro* system provides no evidence for a disfunction of this mRNA as a result of IFN treatment.

DISCUSSION

In the present paper, we have demonstrated differences in the sensitivity to IFN among three MHV strains with distinctly different virulence for mice (Hirano *et al.*, 1981; Taguchi *et al.*, 1981). MHV-2 is a highly virulent virus for adult mice, causing acute hepatitis irrespective of the route of inoculation (Hirano *et al.*, 1975). MHV-S is avirulent for weanling and adult mice and even after intraperitoneal (ip) inoculation with 10^5 PFU, infectious virus can only be found at very low levels in the liver (Taguchi *et al.*, 1977, 1981). MHV-JHM, which has an intermediate virulence, does not cause the death of adult mice, but after ip infection, moderate titers of infectious virus, accompanied by hepatitis, can be detected in the liver (Taguchi *et al.*, 1981). Such differences in virulence among these three MHV strains was initially shown to correlate to the difference in the capacity of the viruses to replicate in mouse macrophages (Taguchi *et al.*, 1983, 1981). We now suggest that a difference in sensitivity to IFN may be an important factor, since

FIG. 3. (a) Comparison of intracellular virus proteins. L cells treated with IFN and infected with MHV strains as mentioned in the legend of Fig. 2a were labeled with [^{35}S]methionine for 30 min before cell harvest at 12 hr p.i. Lysates made as described under Materials were immune precipitated with anti-JHM or normal rabbit serum (NRS). (b) Densinometric analysis of autoradiogram shown in Fig. 3a. A: MHV-2, IFN (+). B: MHV-2, IFN (−). C: MHV-S, IFN (+). D: MHV-S, IFN (−). E: MHV-JHM, IFN (+). F: MHV-JHM IFN (−).

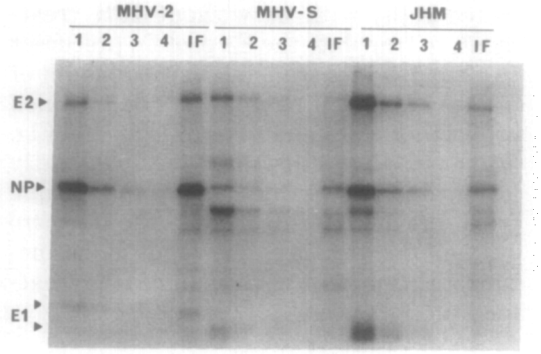


FIG. 4. Quantitative immune precipitation analysis. ^{35}S-labeled cell lysates prepared from IFN-untreated cells as described in the legend of Fig. 3a were diluted 1:1 (1), 1:5 (2), 1:10 (3), and 1:50 (4) and precipitated with anti-JHM rabbit serum together with undiluted (1:1) IFN-treated cell lysate (IF).

there is a strong correlation between the resistance to IFN and virulence for mice. Recently, Garlinghouse *et al.* (1984) reported a slight difference in the sensitivity to IFN among four MHV strains, showing that MHV-S is the most sensitive, MHV-3 the least sensitive, and MHV-JHM and MHV-A59 are of intermediate sensitivity. The data reported by us and those by Garlinghouse *et al.* (1984), together with data on the comparison of virulence of these MHV strains (Hirano *et al.*, 1981), clearly show that the MHV strains which are less sensitive to IFN are more virulent for mice.

It has been reported that the administration of concanavalin A rendered susceptible mice resistant to MHV-2 infection (Weiser and Bang, 1977) and that infection with low-virulent MHV-S, 1 to 2 days prior to challenge with MHV-2, also made the normally susceptible mice resistant (Taguchi *et al.*, 1980). In both these experiments the macrophages taken from pretreated animals were shown to be highly resistant to MHV-2 infection, and the resistance was suggested to be mediated by IFN. In this paper, it was shown that IFN was not effective in suppressing the replication of MHV-2 in tissue culture. Therefore, in the experiments described above, the development of an IFN-mediated antiviral activity of macrophages may protect the many cell types which are susceptible to MHV-2 but are not protected by IFN.

We have begun to analyze the effects of IFN treatment on MHV replication at the molecular level. The experiments showed that the amount of virus-specific mRNAs was significantly reduced by IFN treatment in MHV-S and MHV-JHM infection, but not during MHV-2 infection. This suggests that either mRNA stability, mRNA synthesis, or stages in replication prior to this, e.g., minus-stranded RNA synthesis (Lai *et al.*, 1982), translation of the infectious genomic RNA, or penetration of virions into cells, are sensitive to IFN during MHV-S and MHV-JHM infection.

The effect of IFN on viral proteins during MHV-S and MHV-JHM infections was partly unexpected. As might have been predicted IFN treatment produced an overall reduction of viral proteins. Addi-

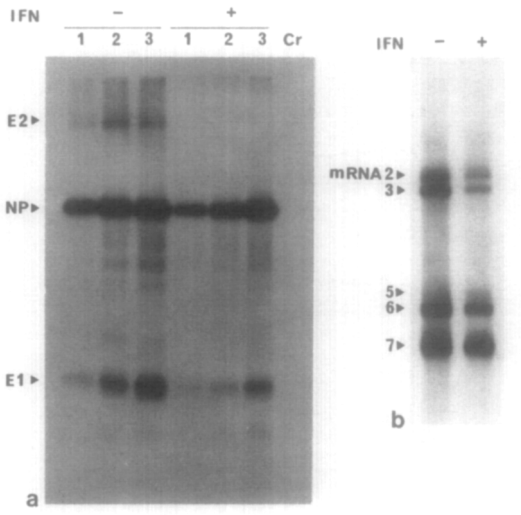


FIG. 5. (a) Cell-free *in vitro* translation. Poly(A) + RNA isolated from MHV-S-infected cells pretreated (+) or untreated (−) with IFN was translated *in vitro* in a rabbit reticulocyte lysate and the products were precipitated with anti-JHM rabbit serum. 0.5 μg (1), 1 μg (2), and 2 μg (3) of poly(A) + RNA containing mRNAs were used for *in vitro* translation. Cr shows the *in vitro* translation with reticulocyte lysate only. (b) The RNA samples were analyzed by Northern blot hybridization to quantitate the amount of mRNA 6 in each sample. mRNA from IFN-treated (+) or -untreated (−).

tionally, however, the viral E1 protein was preferentially reduced in comparison to the other viral proteins. We demonstrated that the mRNA for E1, mRNA 6, isolated from IFN-treated cells was capable at least *in vitro* of translation, but the reduced levels of E1 protein *in vivo* could still result from an IFN-induced block in translation of this mRNA. Alternatively the same result would be seen if the stability of the E1 protein is reduced by IFN treatment. These experiments have demonstrated that there are multiple effects of IFN treatment during MHV infection. Many more experiments will be required to reveal the molecular basis of those effects.

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