*INHIBITION OF SV40 T ANTIGEN FORMATION BY INTERFERON**

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The mechanism of action of interferon has not yet been determined. However, a number of studies[1] have made it clear that its inhibitory effect on virus replication is exerted at a stage following the uncoating of the viral nucleic acid but prior to the replication of the viral genome. Thus it was of interest to determine the effect of interferon on the early prereplicative function of the input viral genome.

The oncogenic DNA virus SV40, which is capable of initiating both lytic and nonlytic infections in mammalian cells, offers a unique opportunity to study this question and also to explore the early events in viral oncogenesis. Cells infected with the SV40 virus or purified SV40 DNA[2] develop a specific antigen (T antigen), detectable by complement fixation and fluorescent antibody (FA), which is distinct from the SV40 virion antigen; it is present in SV40 tumors, transformed cells, and cells acutely infected with SV40 virus.[3–6] In cytolytic infections, the SV40 T antigen is produced early in the infectious cycle prior to the appearance of infectious virus or SV40 virion antigen,[6] and inhibitor studies show that T antigen is produced in undiminished amounts in the presence of inhibitors of viral and cellular DNA synthesis.[7] Thus the SV40 T antigen appears to be coded for by a portion of the input viral DNA and to be formed prior to, and independent of, its replication. Therefore, studies were carried out to determine the effect of interferon on the formation of SV40 T antigen.

In the present studies the 3T3 mouse cell line was employed.[8, 9] With high multiplicities of virus, nearly 100 per cent of these cells are infected and form T antigen, and transformation rates as high as 50 per cent occur without the synthesis of any infectious virus or virion antigen.[10] The absence of viral replication in these cells eliminates the complications attending secondarily infected cells.[11, 12] Inhibition of SV40 transformation of this cell line by interferon has already been demonstrated by Todaro and Baron.[11]

In addition, an interesting ancillary finding is presented, namely, that many of the SV40-infected cells which initially form T antigen lose their ability to produce T antigen with subsequent cell divisions, while nondividing cells retain the T antigen for many days. Only transformed cells appear to retain their T antigen-forming capacity through many generations.

Materials and Methods.—Tissue culture: The origin and properties of the continuous line of mouse fibroblasts (3T3) have been described.[8, 9] The line was obtained from Dr. G. Todaro (New York University) and was used after 11–19 passages in our laboratory. Except where otherwise noted, the cells were grown and maintained in Eagle's minimal essential medium[13] in Earle's balanced salt solution, containing four times the usual concentrations of vitamins and nonessential amino acids, 2 mM glutamine, and penicillin and streptomycin at concentrations of 100 units and 100 μg per ml, respectively (3T3M), and 10% unheated fetal calf serum. In all experiments the cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Primary cultures of African green monkey kidney (AGMK) and the continuous line of *Cercopithecus* kidney cells (BSC-1) were obtained from Microbiological Associates, Inc., and Flow Laboratories, Inc., respectively. Maintenance medium for AGMK cultures consisted of Eagle's basal medium plus 2% heated (56°C for 30 min) agamma calf serum (Hyland Laboratories,

Calif.) with antibiotics. BSC-1 cultures were maintained with 3T3M plus 10% unheated fetal calf serum.

Virus: SV40 strain 777[14] was grown in BSC-1 cells. At the time of maximum cytopathogenic effect (CPE), the cultures were frozen and thawed three times; the crude lysate was clarified by centrifugation at 1500 *g* for 15 min and stored in 1-dram vials at −60°C. The pool used in these experiments titered 2×10^9 tissue culture infectious $doses_{50}$ ($TCID_{50}$) per ml in AGMK roller tube cultures.

Vesicular stomatitis virus (VSV) Indiana strain was grown in primary mouse embryo tissue culture. The pool used titered $10^{6.2}$ $TCID_{50}$ per ml and was stored at −60°C.

Interferon: Mouse serum interferon, produced *in vivo* by the intravenous inoculation of Newcastle disease virus (NDV) and held at pH 2 for 7 days,[15] was kindly supplied by Dr. Samuel Baron (National Institutes of Health). Three batches were used in these experiments; they titered between 1000 and 3000 units per ml by a VSV plaque reduction assay previously described.[15] They were retitrated in each of the present experiments by pretreating tube cultures of 3T3 cells with serial half-log dilutions of interferon for 18 hr and then challenging with 10^3 $TCID_{50}$ of VSV per tube. The tubes were observed daily for 7 days. One unit of interferon is here defined as the amount that protects 50 % of 3T3 tube cultures infected with 10^3 $TCID_{50}$ of VSV. Titers of each batch of interferon by this method agreed with the titers obtained by the plaque reduction assay performed in Dr. Baron's laboratory within the limits of reliability (threefold over-all) reported for the latter.[16]

Control preparations consisted of uninfected mouse serum treated at pH 2 in parallel with interferon preparations.

Detection of SV40 T antigen in 3T3 cells: Confluent monolayer cultures of 3T3 cells in 50-mm plastic Petri dishes (Falcon Plastics, Los Angeles) containing 11 × 22-mm glass coverslips were washed with 3T3M and infected with a multiplicity of 10^3 $TCID_{50}$ of SV40 virus per cell. After 2 hr adsorption at 37°C, 4.5 ml of 3T3M with 5% heated agamma calf serum were added and the cultures returned to 37°C. Control cultures received medium alone. At appropriate times after infection, coverslips were rinsed twice and fixed in cold acetone for 10 min.

In studies to determine the effect of interferon on T antigen formation, confluent monolayer cultures were drained and washed. They were then incubated with appropriate dilutions of interferon or control preparations for 18 hr, washed 3 times with 3T3M, and infected as above. In addition, tube cultures of 3T3 cells were incubated for 18 hr with 1.5 ml of the same dilutions of interferon and control solutions and challenged with 10^3 $TCID_{50}$ of VSV to provide a parallel titration of the interferon preparations.

In one experiment, interferon was added for various time intervals before or after infection with SV40. Some cultures were pretreated for 18 hr as above, while other cultures received interferon after the 2-hr period of virus adsorption or at later time intervals. In the latter instances, the virus inoculum was removed before the addition of the interferon solution and the interferon was left on for the remainder of the experiment.

Coverslips were bisected and stained for the SV40 T antigen with a serum pool from hamsters bearing SV40 tumors. An indirect fluorescent antibody (FA) procedure, the specificity of which has been well established, was used as previously described.[4] Half coverslips from confluent dishes were found to contain approximately 50,000 cells and were read as negative only after all cells were visualized. Positive coverslips were scanned under low power (100×) to verify the uniform distribution of positive nuclei: 100 high-power fields (each containing approximately 50 cells) evenly distributed over the coverslip were then examined. Positive cells were counted in each field and total cells counted in every fifth field to give an estimate of the total number of cells counted. These data, representing high-power visualization of approximately 5000 cells (a randomly distributed 10% of the total cell population), were used to compute the per cent cells with T antigen.

Transformation assay: The colonial morphology transformation assay of SV40-infected 3T3 cells has been described in detail.[9, 10] In experiments to determine the effect of interferon on viral transformation, confluent cultures of 3T3 cells were pretreated with various dilutions of interferon for 18 hr at 37°C, then washed three times and infected with 10^3 $TCID_{50}$ per cell of SV40 virus. After a 2-hr period of adsorption, 4.5 ml of 3T3M with 5% heated agamma calf serum was added, and the cultures were incubated for 20 additional hr at 37°C. The cells in each dish

were then dispersed with trypsin, counted, and 1000 cells in 5 ml 3T3M + 10% fetal calf serum inoculated into each of 10 Falcon Plastic dishes, while 10 dishes were seeded with 200 cells each. These were incubated at 37°C, and fixed and stained with 1% Mayer's hematoxylin at 17 days. In the dishes seeded with 1000 cells, transformed foci could easily be identified against the background of confluent normal cells as dense, darkly stained colonies of piled-up epithelioid cells. Normal 3T3 cells were triangular to fibroblastic and stopped dividing when confluence was achieved. Total colonies as well as transformed colonies were counted in the dishes seeded with 200 cells to determine plating efficiency. Transformation rates are expressed as the ratio of transformed colonies to both the total number of colonies that appeared (*t*-col) and the number of cells that were plated (*t*-cell). Since transformation rates were approximately the same in both groups, the transformation rates in 200 and 1000 cell dishes were pooled for presentation. Uninfected cultures were trypsinized and similarly plated for determination of plating efficiency. No transformed colonies were seen in uninfected cultures.

Results.—Effect of interferon on the time course of SV40 T antigen formation: In Figure 1, the time course of SV40 T antigen formation in control and interferon pretreated 3T3 cells is shown. Cells containing T antigen were first observed at 6 hr in control cultures. The staining was characteristic of the SV40 T antigen in that it was granular, confined to the nucleus, and spared the nucleoli.[4, 5] The per cent positive cells was at or near maximum by 48 hr and, in these confluent nondividing cultures, remained maximum without visible loss of intensity for at least 7 days. In contrast, cells pretreated with 100 units per ml of mouse serum interferon had no detectable T antigen at 12 hr after infection. As judged by the displacement of the curve of T antigen formation, there was a delay of 10–12 hr in T antigen production in the interferon-pretreated cultures. Also, there was approximately an 84 per cent reduction in the per cent of T-antigen-positive cells at 48 hr and even 7 days later there was a significant reduction in the number of cells containing T antigen in the interferon-treated cultures. Thus interferon appears to reduce the number of cells that produce T antigen after high-multiplicity infection with SV40 virus and delay T antigen production in those cells which ultimately do form T antigen.

Sensitivity of SV40 T antigen production to interferon: Table 1 presents the results of three groups of experiments in which 3T3 cultures were pretreated with varying doses of interferon and control preparations, and subsequently infected with 10^3 $TCID_{50}$ SV40 virus per cell. It is clear that in all experiments, and at all time intervals examined, pretreatment with interferon markedly inhibited T antigen production. The data obtained 48 hr after infection in experiment 2 are plotted as per cent inhibition versus interferon dose (Fig. 2, curve *A*). It is noteworthy that 50 per cent inhibition of T antigen formation occurred at interferon concentrations of approximately 1 unit per ml, indicating that this expression of the SV40 genome is approximately as interferon-sensitive as is VSV lytic infection.

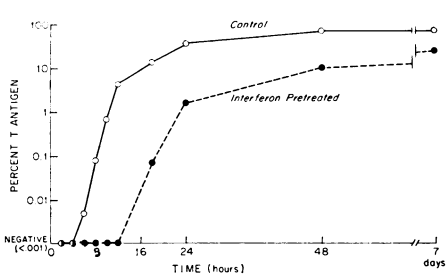

FIG. 1.—Effect of interferon on the time course of T antigen formation in 3T3 cells infected with SV40 virus. Monolayer cultures of 3T3 cells were pretreated for 18 hr at 37°C with 100 units of mouse serum interferon per ml or control preparations. The cultures were infected at time 0 with a multiplicity of 10^3 $TCID_{50}$ of SV40 virus per cell. Coverslips were acetone-fixed at the indicated times and stained for the SV40 T antigen by indirect FA. They were read as negative when no T-antigen-positive cells were seen in 10^5 cells (<0.001%).

TABLE 1

INHIBITION OF SV40* T ANTIGEN PRODUCTION IN 3T3 CELLS BY INTERFERON

Expt. no.	Interferon preparation	Interferon dose† (units/ml)	Per Cent Cells with T Antigen‡ 24 hr	48 hr	88 hr	7 days	Per Cent of Control 24 hr	48 hr	7 days
1	A	100	1	10			1	10	
		30	5				6		
		10	7				8		
		3	12				14		
		Control§	84	97	95		100	100	
2	B	100		4		8		7	15
		30		8				13	
		10		12				20	
		3		13				21	
		1		23				39	
		Control§		61		56		100	100
3	C	100	1	10		25	3	16	36
		Control§	37	64		70	100	100	100

* Infected at time 0 with 10^3 $TCID_{50}$ of SV40 virus per cell.
† 3T3 cultures pretreated with 3 ml interferon solutions for 18 hr at 37°C.
‡ Uninfected controls were always negative when stained for T antigen.
§ pH 2 treated and untreated mouse serum controls produced no inhibition of T antigen.

Inhibition of SV40 T antigen by interferon applied for various time intervals before and after infection: The results of experiments involving the addition of 100 units per ml interferon at different times before and after SV40 infection of confluent nondividing 3T3 cultures are presented in Table 2. Interferon produced significant inhibition of T antigen formation when added prior to, or up to 2 hr after, SV40 infection. However, there was no detectable inhibition when interferon was added 4 hr after infection or at any time thereafter. The time required for exogenous interferon to establish the antiviral state within the cells is not known for this system. However, the fact that 100 units per ml of interferon added 2 hr after infection produced a degree of T antigen inhibition (39%) less than that seen when cultures were pretreated for 18 hr with only 1 unit per ml (Fig. 2), and the failure of interferon added at 4 hr to produce any detectable inhibition, suggests that the interferon-sensitive biochemical reaction in the synthesis of T antigen is completed sometime between 2 and 4 hr after infection.

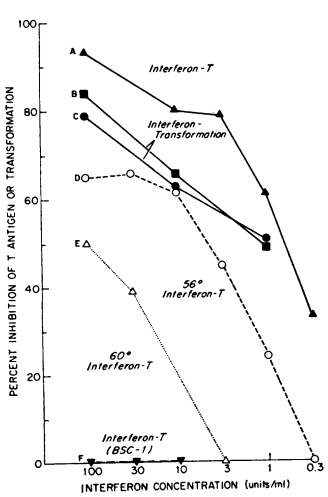

FIG. 2.—Effect of interferon on T antigen formation and transformation in 3T3 cells infected with SV40 virus. All cultures were pretreated with mouse serum interferon or control preparations for 18 hr at 37°C and then infected with a multiplicity of 10^3 $TCID_{50}$ of SV40 virus per cell. Curve *A* (▲——▲), per cent inhibition of T antigen formation by mouse serum interferon; curve *B* (■——■), per cent inhibition of transformation by mouse serum interferon (*t*-cell); curve *C* (●——●); per cent inhibition of transformation by mouse serum interferon (*t*-col); curve *D* (O- - -O), per cent inhibition of T antigen formation by interferon heated 56° 1 hr; curve *E* (△. . .△), per cent inhibition of T antigen formation by interferon heated 60° 1 hr; curve *F* (▼-·-·-▼), per cent inhibition of T antigen formation by mouse serum interferon in heterologous cells (BSC-1). One unit of interferon is defined as that amount producing 50% inhibition of VSV lytic infection in the same experiment. Titer of interferon preparation used was 1000 units/ml. Titer after 56° heating was 100 units/ml and after 60° heating was 10 units/ml.

TABLE 2

INHIBITION OF SV40 T ANTIGEN IN 3T3 CELLS BY INTERFERON APPLIED FOR VARIOUS INTERVALS OF TIME BEFORE AND AFTER VIRUS INOCULATION

Interval of Contact with 100 Units per ml Interferon* Time interferon added (hr)	Time interferon removed (hr)	Per cent T antigen at 48 hr	Per cent inhibition
−18†	48	9	87
−18	0	10	85
−1	0	37	44
+2	48	40	39
+4	48	70	0
+6	48	67	0
+8	48	70	0
+10	48	71	0
+16	48	67	0
Control	—	66	—
Uninfected control	—	0	—

* Interferon (100 units per ml) added in 3 ml of 3T3M + 5% heated agamma calf serum. 3T3 cultures infected at time 0 with 10^3 $TCID_{50}$ of SV40 virus per cell. Experiment terminated with fixation of coverslips at +48 hr.
† Interferon removed from these cultures at time of infection (time 0) and re-added at +2 hr.

Comparison of the effect of interferon on T antigen production and transformation in 3T3 cells infected with SV40: The results of transformation assays done in parallel with T antigen determinations (Table 1, expt. 2) are presented in Table 3. Preincubation for 18 hr with 100 units of interferon per ml produced 79 per cent inhibition of transformation in cultures infected with 10^3 $TCID_{50}$ SV40 virus per cell. It is again evident that 1 unit per ml of interferon produced approximately 50 per cent inhibition. The data for T antigen inhibition and inhibition of transformation from the same experiment are plotted in Figure 2 (curves *A*, *B*, and *C*) as per cent inhibition versus interferon dose. It is noteworthy that the sensitivity to interferon and slope of the interferon dose response curves of transformation and T antigen production are quite similar, suggesting that interferon inhibits a single function necessary for both these expressions of the SV40 viral genome.

Characterization of the inhibitor of SV40 T antigen as interferon: Since all interferon preparations were treated at pH 2 for 7 days, the inhibitor of T antigen formation studied here was pH 2 stable. In the experiments described, cultures were preincubated with interferon and then thoroughly washed prior to SV40 infection. This precluded inhibition by an inhibitor that, like antibody, interacts directly with extracellular virus. In addition, data presented in Table 2 show that the interferon preparations were able to inhibit T antigen formation when added 2 hr after infection.

TABLE 3

INHIBITION OF SV40* TRANSFORMATION OF 3T3 CELLS BY INTERFERON

Interferon dose† (units/ml)	*t*-Cell‡	*t*-Col§	Plating efficiency (%)	Per Cent Control — Based on *t*-Cell	Per Cent Control — Based on *t*-Col
100	0.8	6	15	16	21
10	1.8	10	18	34	37
1	2.6	13	21	51	49
0	5.1	26	21	100	100
Uninfected control	0	0	26	—	—

* 3T3 cultures infected with 10^3 $TCID_{50}$ of SV40 virus per cell.
† 3T3 cultures pretreated with 3 ml interferon solution for 18 hr at 37°C.
‡ *t*-Cell represents the per cent of cells plated which yielded transformed colonies. Each point represents data from 12 × 10^3 plated cells.
§ *t*-Col represents the per cent of total colonies formed that were transformed.

Samples of interferon preparation B heated at 56°C or 60°C for 1 hr, preincubated with antibody to NDV, or centrifuged at 105,000 *g* for 3 hr were assayed in parallel for their ability to inhibit T antigen formation and to protect against VSV lytic infection. The preparations treated with antibody to NDV and centrifuged for 3 hr at 105,000 *g* showed no loss of inhibitory activity, either against SV40 T antigen or VSV. However, the ability of the interferon preparation to inhibit both T antigen formation and VSV lytic infection was partially destroyed by heating for 1 hr at 56°C (Fig. 2, curve *D*) and more extensively inactivated by heating at 60°C for 1 hr (curve *E*). It was also found that mouse serum interferon failed to inhibit T antigen formation by SV40 virus in heterologous cells (i.e., BSC-1 line of *Cercopithecus* kidney cells) (Fig. 2, curve *F*). Several pH 2 treated and untreated normal mouse sera were used as controls. None of these produced any reduction in T antigen formation. These results demonstrate that the factor in the mouse serum interferon preparations that inhibits SV40 T antigen formation apears to have the properties of interferon.

Persistence of T antigen in dividing and nondividing cells: In conjunction with some of the above experiments, cells were trypsinized 20 hr after SV40 infection, seeded on coverslips at concentrations of 10^3 and 10^6 cells per dish, and FA stained at various times for T antigen. At time of trypsinization, a high but not maximal per cent of cells were positive for T antigen (compare Table 1 and Fig. 1) and 24 hr later, both sets of trypsinized cells showed the same per cent T antigen staining (approximately 85%) as untrypsinized infected controls. However, 7 and 11 days after trypsinization the heavily and lightly seeded cultures were markedly different. Those seeded with 10^6 cells, in which there was little or no cell division, had undiminished per cent and intensity of T antigen staining. In contrast, the lightly seeded cultures, which had undergone approximately 10 generations, showed no detectable T antigen except in occasional focal areas which appeared to represent transformed colonies. Thus, SV40 T antigen appears to be lost from dividing cells unless some particular event, perhaps the integration of the SV40 genome, occurs to perpetuate the T antigen producing potential in the cell.

Discussion.—A number of observations indicate that T antigen formation reflects the presence and function of SV40 genetic material, rather than derepression of the host cell genome,[2–6, 17–20] and that T antigen formation is an early function of the input SV40 genome independent of its replication.[2, 7] Thus the finding that interferon inhibits SV40 T antigen production demonstrates that the action of interferon involves inhibition of the early function of the input SV40 DNA.

Recent work with RNA viruses[21] has shown that interferon (or more correctly the interferon-induced intracellular protein mediating its action)[22] inhibits the association of input viral RNA with host cell ribosomes, preventing the formation of the viral polysomes presumably required for synthesis of early viral proteins. Thus, for RNA viruses, there is also evidence of inhibition by interferon of the early function of the input viral genome.

The fact that both SV40 T antigen production and SV40 transformation in 3T3 cells are sensitive to interferon with very similar dose-response curves suggests the inhibition by interferon of a single event necessary for both these expressions of the viral genome. It should also be noted that both these expressions of the SV40 viral genome are approximately as sensitive to interferon as is VSV lytic infection.

Although this similarity may be coincidental, it suggests that the mechanism of action of interferon is the same in these widely different cell-virus interactions. It seems probable from the data presented above that interferon produces its antiviral action either by inhibiting the synthesis of virus-specific messenger RNA (mRNA) on the input viral template or by blocking the function of the virus-specific mRNA. The similar interferon sensitivity of an RNA virus (VSV) and a DNA virus (SV40) observed in these experiments favors the latter mode of action.

Although the interferon sensitivity of T antigen formation and transformation are similar in confluent cultures of 3T3 cells, the effect of cell division on these two expressions of the SV40 viral genome and on their sensitivity to interferon is, at least superficially, quite different. It has been demonstrated by Todaro and Green that cell growth is necessary for the establishment of transformation in SV40-infected 3T3 cells and that the interferon sensitivity of transformation is lost within 18 hr in dividing cultures but persists for more than 4 days in confluent nondividing cultures.[23] In contrast, even in confluent nondividing cultures, T antigen formation becomes completely insensitive to interferon 4 hr after SV40 infection; also, SV40 transformed cells that are T-antigen-positive remain so throughout hundreds of generations in tissue culture, even in the continuous presence of interferon.[24] Finally, SV40 T antigen persists for more than 7 days in confluent nondividing cells but is entirely lost from dividing cells (except for occasional colonies of probably transformed cells) within several divisions.

These data indicate that the SV40 genome has both an integrated and a nonintegrated relationship with 3T3 cells. T antigen is produced when the viral genome is in either the integrated or the nonintegrated state. In contrast, transformation appears to occur only when the SV40 genome is integrated. In the majority of acutely infected (T-antigen-positive) cells, the viral genome is not integrated and is lost with cell division, the progeny being T-antigen-negative and not transformed. In a proportion of infected cells, however, the viral genome becomes integrated, presumably at the time of DNA replication or cell division.[23, 25] With integration, T antigen formation becomes stable and heritable, and the cells are transformed. In such cells, T antigen formation cannot be blocked by interferon.

It is striking that a prereplicative viral synthetic process (T antigen production) whose initiation is interferon-sensitive, becomes resistant to interferon when the viral genome is in the integrated state. However, it is not yet known whether, once initiated, T antigen formation by 3T3 cells with virus in the nonintegrated state can be blocked by interferon. If preformed T antigen is relatively stable in nondividing cells, subsequent inhibition of its synthesis by interferon may be obscured. The question of the stability of preformed T antigen is also of central importance to an understanding of the apparent disparity between the findings that in nondividing 3T3 cells the interferon sensitivity of T antigen formation is lost by 4 hr, while SV40 transformation remains sensitive to interferon for at least 4 days.

It is clear that interferon does block integration of the SV40 genome. Whether it does this without also blocking the T antigen synthetic activity of the nonintegrated SV40 genome is presently under study.

Summary.—Pretreatment of mouse fibroblast (3T3) cells with interferon reduced the number of cells that produced SV40 T antigen after infection with SV40 virus and resulted in a delay of T antigen formation in ultimately positive cells. This

sensitivity to interferon was lost by 4 hr after viral infection. Transformation of 3T3 cells by SV40 was as sensitive to the inhibitory action of interferon as T antigen formation, suggesting that interferon inhibits a single function necessary for both of these expressions of the SV40 viral genome. The results indicate that interferon acts by inhibiting an early function of the input viral DNA.

The SV40 T antigen was found to be stable in nondividing and tranformed cells, but was lost with division in nontransformed cells. The hypothesis was advanced that two alternative virus-cell associations may exist in SV40-infected 3T3 cells, with the SV40 genome coding for T antigen in both. In one case the viral genome is integrated and interferon-resistant, resulting in transformation and perpetuation of T antigen formation through succeeding generations. In the other, the SV40 genome is not integrated and remains interferon-sensitive; transformation does not occur and T antigen formation is lost with cell division.

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