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Hypersensitivity to Hg^{2+} and Hyperbinding Activity Associated with Cloned Fragments of the Mercurial Resistance Operon of Plasmid NR1

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The region of plasmid NR1 concerned with resistance to Hg^{2+} and organomercurials consists of sequences found on restriction endonuclease fragments *Eco*RI-H and *Eco*RI-I. When both fragments were cloned together into a derivative of plasmid ColE1, the hybrid plasmid conferred properties indistinguishable from those of the parental plasmid, NR1: resistance to Hg^{2+} and to the organomercurials merbromin and fluoresceinmercuric acetate and the inducible synthesis of the enzyme mercuric reductase. When fragment *Eco*RI-I was cloned into plasmid ColE1, cells containing the plasmid were as sensitive to Hg^{2+} and organomercurials as plasmidless strains. When fragment *Eco*RI-H was cloned into ColE1, cells with the hybrid plasmid were hypersensitive to Hg^{2+} and organomercurials. This hypersensitivity was inducible by prior exposure to low, subtoxic Hg^{2+} or merbromin levels. It was associated with an inducible hyperbinding activity attributed to a gene governing Hg^{2+} uptake and found on fragment *Eco*RI-H (which contains the proximal portion of a mercuric resistance [*mer*] operon).

Mercuric salt and organomercurial resistance is determined by genes on about 25% of all enteric bacterial antibiotic resistance plasmids (22), including the classic R factor NR1 (also known as R100 or R222). The basis for Hg^{2+} resistance is the volatilization of mercury from the growth medium (22, 25, 29, 30). Volatilization is brought about by the enzyme mercuric reductase, a flavin adenine dinucleotide-containing flavoprotein that is an NADPH-dependent oxidoreductase (23). The synthesis of this enzyme and its associated resistance are strictly inducible by subtoxic levels of Hg^{2+} or organomercurials (22, 29, 30). In this paper, we report on the properties of cells harboring hybrid plasmids containing the vector plasmid ColE1 plus restriction endonuclease *Eco*RI fragments, known from previous studies (5, 15, 18, 27) to come from the *mer* region. These studies have proved complementary to those in the accompanying papers (10, 11) on *mer* mutants. They define a new gene (*merT*) governing the Hg^{2+} uptake process.

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MATERIALS AND METHODS

Escherichia coli strain KP245 *met his trp thy lac gal* (18) was used as host for the hybrid plasmids, which consisted of the ColE1::Tn*A* plasmid vector into which *Eco*RI fragments from plasmid NR1 were cloned. These hybrid plasmids were pRR130 (with *Eco*RI-H plus *Eco*RI-I fragments of NR1), pRR132 (with *Eco*RI fragment I), and pRR134 (with *Eco*RI fragment H) (18). Strain JG121 with the parental plasmid NR1 or with plasmid pRR35 (a mutant plasmid with Tn*3* inserted into the *merA* gene of plasmid NR1) was also used.

Overnight cultures in nutrient broth (Difco) were diluted and grown in tryptone broth (22, 23). In most experiments, induction of the *mer* system was accomplished by the addition of low levels of Hg^{2+} or merbromin (as indicated below) when the cultures had reached 75 Klett units of turbidity (Klett colorimeter with no. 54 filter; equivalent to 0.17 mg [dry weight] per ml), followed by an additional 60 to 90 min of incubation at 37°C. After centrifugation and resuspension of the cells, binding of $^{203}Hg^{2+}$ was measured in tryptone broth containing 100 μM EDTA (which appeared to prevent nonbiological loss of $^{203}Hg^{2+}$ from solution), 100 μM $MgSO_4$, and 100 μg of chloramphenicol per ml (to prevent induction during the assay period). Samples were removed periodically, filtered on membrane filters (type HA; Millipore Corp.), and washed twice with 5 ml of broth. The samples were counted by liquid scintillation spectroscopy. The rate

of volatilization of 5 μM $^{203}Hg^{2+}$ from phosphate assay buffer under conditions of rapid shaking was measured as previously described (19, 23, 29).

RESULTS

The recombinant plasmids used in this study have recently been described; pRR130 is a recombinant ColE1::Tn*A* plasmid with cloned *Eco*RI fragments H and I from plasmid NR1 (18). It confers all of the mercurial resistances associated with plasmid NR1 (18, 19; see below). Plasmids pRR134 (ColE1::Tn*A* with cloned *Eco*RI-H of NR1) and pRR132 (ColE1::Tn*A* with cloned *Eco*RI-I of NR1) have been described previously (18) and do not confer mercurial resistance. Cells harboring plasmid pRR134 were in fact hypersensitive toward Hg^{2+} in liquid growth experiments when compared with the isogenic strain carrying plasmid pRR132 (Fig. 1) or without a plasmid (data not shown). The hypersensitivity was toward Hg^{2+} and toward the organomercurials merbromin, fluoresceinmercuric acetate, and phenylmercuric acetate (data not shown). *E. coli* K-12 carrying pRR130 showed resistances and sensitivities to organomercurials identical to those of *E. coli* K-12 with plasmid NR1 (R222 in reference 30).

Sensitivity, resistance, and hypersensitivity were measured in experiments such as those shown in Fig. 1 by growth over periods of hours. Mercury inhibited the incorporation of [^{14}C]-uracil into trichloroacetic acid-precipitable material during a 10-min period (Fig. 2). In these short-term experiments, somewhat higher Hg^{2+} levels were needed to demonstrate differential resistance or sensitivity. With chloramphenicol added to prevent induction of the *mer* system during the period of [^{14}C]uracil incorporation, hypersensitivity of the strain with plasmid pRR134 to Hg^{2+} was dependent upon previous induction by subtoxic 0.25 μM Hg^{2+} (Fig. 2). Without preinduction, cells carrying plasmid pRR134 were no more sensitive to Hg^{2+} than were cells carrying plasmid pRR132. Thus, hypersensitivity to Hg^{2+} is inducible. Analogous experiments demonstrated an inducible hypersensitivity in strain KP245(pRR134) for the rate of protein synthesis (by measurement of incorporation of [^{3}H]leucine into acid-precipitable material; data not shown). Experiments such as the one in Fig. 2 do not distinguish between inhibition at the level of uracil uptake and inhibition of subsequent RNA synthesis. However, since the purpose of the experiment was to demonstrate inducible hypersensitivity to Hg^{2+}, the basis of the inhibition was not pursued.

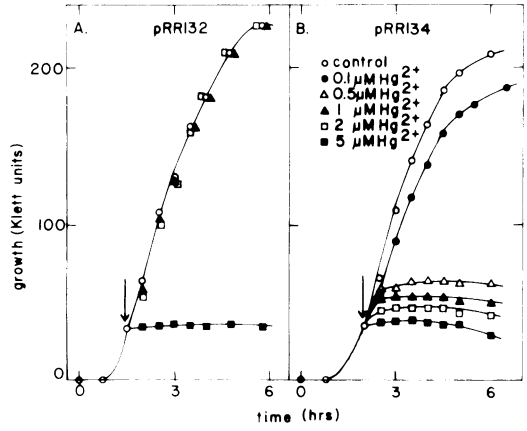

FIG. 1. *Inhibition of growth by Hg^{2+}. Strains KP245(pRR132) (sensitive) (A) and KP245(pRR134) (hypersensitive) (B) were grown in Difco nutrient broth at 37°C to 30 to 35 Klett units of turbidity. The cultures were divided, varying concentrations of $HgCl_2$ were added, and growth was monitored in a Klett colorimeter.*

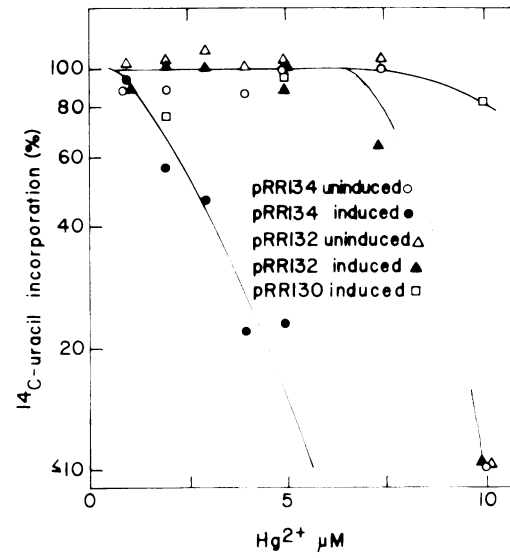

FIG. 2. *Hg^{2+} inhibition of RNA synthesis. Strains KP245(pRR130) (resistant), KP245(pRR132) (sensitive), and KP245(pRR134) (hypersensitive) were grown in tryptone broth to 0.14 mg (dry weight) per ml and induced (or not) by addition of 0.25 μM Hg^{2+}. After a subsequent cell doubling, the cultures were harvested by centrifugation, and the cells were suspended in tryptone broth containing 100 μg of chloramphenicol per ml (to prevent induction during the assays). [^{14}C]uracil (0.04 μCi/ml) was added, and samples were removed to ice-cold 10% trichloroacetic acid at 10 min. After 1 h on ice, the precipitates were filtered, washed, and counted by liquid scintillation spectroscopy. The data are shown normalized to the controls without Hg^{2+} with each strain (which represented about 5% incorporation of available uracil).*

Cellular binding of mercury. A hypothesis for hypersensitivity to Hg^{2+} which is consistent with the results with hypersensitive *merA* mutants (11) is that the hypersensitive cells bound appreciably more Hg^{2+} at exposure levels which distinguished between the hypersensitive and sensitive strains. Figure 3A shows the greater binding of mercury from a 2 μM Hg^{2+} solution by the induced hypersensitive cells than by uninduced cells of the hypersensitive strain with plasmid pRR134 or by cells of the sensitive strain with plasmid pRR132. The total $^{203}Hg^{2+}$ level in these cultures remained constant during the experimental period. With resistant cells, mercury was lost by volatilization, and still less mercury was bound to the cells (data not shown). The difference between mercury binding by the hypersensitive and the sensitive cells was found only at (or below) those Hg^{2+} concentrations that distinguish their resistance levels. At Hg^{2+} concentrations above 10 to 20 μM Hg^{2+} (which cause rapid inhibition of the metabolism of both strains), the sensitive cells bound increasingly more Hg^{2+}, and both sensitive and hypersensitive cells showed saturation of binding at approximately the same level (Fig. 3B).

Since plasmid pRR134 conferred hypersensitivity to merbromin and fluoresceinmercuric acetate as well as to Hg^{2+}, we tested the effects of the organomercurials on $^{203}Hg^{2+}$ binding and the binding of the nonradioactive organomercurials by fluorescence measurements in an Aminco filter fluorimeter. Fluoresceinmercuric acetate inhibited Hg^{2+} binding by induced hypersensitive cells much more effectively than did merbromin, with approximately 25 μM fluoresceinmercuric acetate or 700 μM merbromin required to inhibit the binding of 2 μM Hg^{2+} by 50%. However, neither fluoresceinmercuric acetate nor merbromin bound to induced hypersensitive cells in appreciable amounts, and we were unable to detect differences in the binding of these organomercurials among resistant, sensitive, and hypersensitive cells (data not shown). At concentrations of 25 to 100 μM fluoresceinmercuric acetate or merbromin, less than 0.2% of the organomercurials bound rapidly to the cells. The relatively lower binding of the organomercurials in these experiments is consistent with the relatively lower toxicity compared with Hg^{2+} seen in our current and earlier studies (29, 30).

Experiments to test the energy dependence or reversibility of Hg^{2+} binding by sensitive and hypersensitive cells have provided some preliminary information. Incubation at 4°C resulted in appreciably less uptake of $^{203}Hg^{2+}$ by both sensitive and hypersensitive cells (data not shown). Carbonylcyanide *m*-chlorophenylhydrazone at 100 or 200 μM inhibited the initial binding of $^{203}Hg^{2+}$ by induced hypersensitive cells. However, 1 mM NaCN was essentially without effect on Hg^{2+} uptake.

Mercuric ions and mercurials bind tightly to thiol groups. The thiol reagents cysteine and β-mercaptoethanol (at 100 μM each) had essentially no effect on the uptake of 2 μM Hg^{2+} by the hypersensitive cells, although 100 μM dithiothreitol had a somewhat inhibitory effect. Addition of these thiol reagents at concentrations up to 1 mM eluted less than 30% of the $^{203}Hg^{2+}$ bound by hypersensitive cells during 20 min. Clearly the cellular binding was highly specific and tight. The bound $^{203}Hg^{2+}$ was precipitated

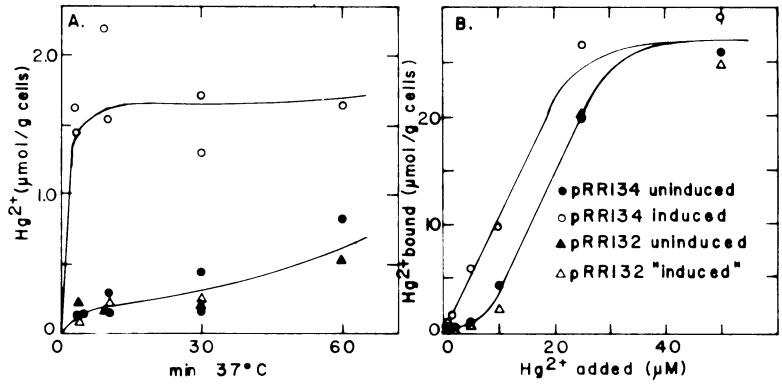

FIG. 3. *Inducible hyperbinding of Hg^{2+} and saturation of Hg^{2+} binding sites. Strains KP245(pRR132) (sensitive) and KP245(pRR134) (hypersensitive) were grown, induced or not by addition of 0.20 μM Hg^{2+} 60 min before harvesting, centrifuged, and suspended in chloramphenicol-containing broth as described in the legend to Fig. 2. (A) Induction of binding activity. $^{203}Hg^{2+}$ at 2 μM was added, and cell-bound mercury was measured periodically by membrane filtration and washing with broth. (B) Saturation of binding sites. $^{203}Hg^{2+}$ was added, and samples were filtered and washed as in (A). The average values for the 5- and 10-min data are shown.*

with the cells by cold trichloroacetic acid (data not shown). Even after heating the cells at 95°C in a solution containing 75 mM Tris-hydrochloride (pH 6.8), 3 mM EDTA, and 2% (wt/vol) sodium dodecylsulfate, 66% of the bound radioactivity remained precipitable by trichloroacetic acid. However, when 190 mM β-mercaptoethanol was added to this buffer before heating (conditions generally used to dissolve cellular protein before gel electrophoresis), all of the $^{203}Hg^{2+}$ was rendered soluble in trichloroacetic acid, although no volatilization of mercury occurred.

The distribution of $^{203}Hg^{2+}$ bound to sensitive and to hypersensitive cells was analyzed by using French press disruption and low-speed and high-speed centrifugation. Although the induced hypersensitive cells bound about four times as much $^{203}Hg^{2+}$ as the uninduced hypersensitive or sensitive cells, there was no significant difference in the distribution of radioactivity. Most of the $^{203}Hg^{2+}$ was bound to particulate material, and most of that was found in the pellet after high-speed (140,000 × *g*) centrifugation (data not shown). This pellet contains both membrane material and ribosomes. A somewhat different distribution of $^{203}Hg^{2+}$ bound to a mercuric-resistant pseudomonad (28) and of $^{115m}Cd^{2+}$ bound by cadmium-resistant *S. aureus* (24) was found in earlier studies.

If a membrane uptake system is required to bring Hg^{2+} to intracellular binding sites, then reagents that break down the cellular permeability barriers would be expected to increase mercury binding. Consistent with this hypothesis, cells pretreated with toluene or sodium deoxycholate took up more $^{203}Hg^{2+}$ than uninduced cells and eventually as much as preinduced hypersensitive cells (data not shown). Furthermore, cells killed by heating to 100°C bound approximately the same amount of mercury as live hypersensitive cells (data not shown).

Pretreatment of the hypersensitive induced cells with 1 mM *N*-ethylmaleimide followed by 10 mM mercaptoethanol (conditions of reference 12) and washing caused only a 20 to 40% decrease in $^{203}Hg^{2+}$ binding by induced hypersensitive cells, although *N*-ethylmaleimide treatment inhibited [^{14}C]uracil uptake by at least 90% (data not shown). If surface thiol groups are involved in mercury uptake, these are clearly of an unusual (and buried) nature.

Induction of binding activity. Hg^{2+} was used in most of the experiments at a concentration (0.20 μM) that maximally induced binding activity of the hypersensitive strains without inhibiting cell growth. In other experiments, we tested whether hyperbinding activity and mercury volatilization activity were coordinately induced with Hg^{2+} or merbromin in the hypersensitive strain (with pRR134) and the resistant strain (with pRR130). The results showed approximately coordinate induction (Fig. 4) as expected, but the induction threshold of inducer concentration varied from experiment to experiment by as much as a factor of 10. Hg^{2+} consistently induced synthesis of both binding activity and volatilization activity at lower concentrations than needed with the gratuitous inducer merbromin.

DISCUSSION

The genetic model in an accompanying paper (11) provides a basis for understanding the results with cells hypersensitive to mercuric ion. The determinants of Hg^{2+} and organomercurial resistance occur entirely on *Eco*RI fragments H and I (18), since plasmid pRR130 with both cloned fragments has all of the characteristics of plasmid NR1 with regard to Hg^{2+} and organomercurial resistance. The structural gene for mercuric reductase is apparently cleaved by restriction endonuclease *Eco*RI. Thus, neither cloned fragment confers resistance or enzyme production. Fragment *Eco*RI-I does not have a measurable phenotype because it lacks an intact *mer* gene or a site of initiation of *mer*-specific RNA synthesis. However, it does have part of the *merA* gene that can recombine with mutants

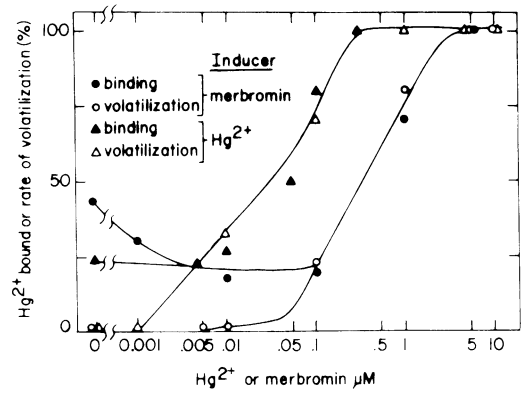

FIG. 4. *Induction of mercuric reductase and hyperbinding activity. Strains KP245(pRR130) (resistant) and KP245(pRR134) (hypersensitive) were grown in broth to 0.17 mg (dry weight) per ml. The cultures were divided, and* Hg^{2+} (△,▲) *or merbromin* (○,●) *was added. After 60 min of incubation at 37°C, when the cell mass had increased to about 0.28 mg/ml, the cells were harvested by centrifugation. KP245(pRR134)* (●,▲) *was assayed for binding of 2 μM* $^{203}Hg^{2+}$ *in broth containing EDTA,* Mg^{2+}, *and chloramphenicol. The average values for the 5- and 10-min results in two independent experiments are shown. KP245(pRR130)* (○,△) *was assayed for the rate of volatilization of 5 μM* $^{203}Hg^{2+}$ *in assay buffer containing 100 μg of chloramphenicol per ml.*

defective in that region (11). The *Eco*RI-H fragment contains the operator-proximal portion of the *mer* operon, the regulatory gene *merR* and the *merT* gene. The regulatory *merR* gene is responsible for the inducible functioning of the *merT* gene, whose product is involved in the uptake of Hg^{2+}. The result of a functional Hg^{2+} transport system without a functional reductase enzyme is to confer hypersensitivity to Hg^{2+} and to organomercurials.

The plasmid-determined enzyme mercuric reductase, which converts toxic Hg^{2+} to less toxic and volatile Hg^0, is strictly intracellular by all measurements (23, 26; unpublished data) and requires the intracellular cofactor NADPH (23). Therefore, the cells have the problem of bringing the toxic extracellular substrate to the intracellular enzyme without poisoning themselves. A close association of the enzyme to a membrane transport system appears to be an answer, but we have never found evidence for a membrane-associated form of the enzyme (26; unpublished data).

A transport process involved in an enzymatic detoxification mechanism is not without precedent. An example is the resistance to streptomycin governed by antibiotic resistance plasmids (including NR1). Here, however, there is a considerable difference, since the adenylylation of a small fraction of the available streptomycin (with the intracellular cofactor ATP) blocks entrance for the major portion of the added antibiotic (4, 6). With the mercuric reductase system, most of the Hg^{2+} is reduced and volatilized (22, 30).

Our efforts to characterize the process of Hg^{2+} uptake and the nature of the binding sites have not been very informative. Total Hg^{2+}-binding capacities of the sensitive and hypersensitive cells were approximately the same (Fig. 3B). The cells differed, however, in the uptake of low levels of Hg^{2+} by a high-affinity process. The ultimate binding sites for Hg^{2+} appear to be unrelated to *mer* operon function and primarily intracellular, since membrane-perturbing agents stimulate mercury uptake by uninduced cells and since thiol reagents, such as *N*-ethylmaleimide, do not affect mercury binding. At first glance, it seems surprising that the *mer*-specified surface receptor protein for a substrate like Hg^{2+} would not involve titratable thiol groupings. Yet it may in fact be specifically important for its normal function that this protein be free of chemical ligands that would immobilize Hg^{2+} and mercurials. The preponderance of evidence favors a *merT* gene-governed uptake system that is inhibited by cold temperatures and by energy uncouplers.

One further line of experimentation suggested the existence of a Hg^{2+} uptake system; in our recent study of effects of gene copy number on Hg^{2+} resistance and mercury volatilization, we found important differences in volatilization kinetics between intact cells and the mercuric reductase enzyme that could be isolated from them (19). Especially with high-gene-copy-number plasmids such as pRR130, we found that much of the mercuric reductase activity was cryptic, as if a rate-limiting uptake pathway prevented the cells from volatilizing mercury as rapidly as the enzyme could function. This cryptic enzyme could be exposed by rupture of the cells or by disruption of the membrane integrity by toluene or deoxycholate.

Hg^{2+} toxicity seems to affect various aspects of cell function. Inhibition of [^{14}C]uracil and [^{3}H]leucine incorporation was demonstrated in our experiments. In contrast, respiration was relatively resistant to Hg^{2+} in our experiments (13). Macromolecular synthesis might be particularly sensitive to Hg^{2+} poisoning, either directly due to effects on synthesis of precursors, binding to polynucleotides (3), or inhibition of assembly enzymes, or indirectly due to release of a degradative RNAse activity (2). Many enzymes containing critical thiol groupings are also very sensitive to mercury and mercurials in vitro (7, 17). These include membrane-bound enzymes, such as the oxidative phosphorylation adenosine triphosphatase (1) and components of amino acid transport systems (14, 21).

Indeed, mercury has been shown to affect membrane permeability of yeast cells (20). Hg^{2+} and the organomercurials toward which plasmids confer resistance are extensively used as heavy atom labels for X-ray crystallography of proteins (16). Considering the multiplicity of potential binding sites and sites for mercurial toxicity, it seems ill advised to seek the target of mercury hypersensitivity at the physiological level. Rather, our future work will be directed toward identification of the protein product of the *merT* uptake system, for example by gel electrophoretic analysis of membrane proteins from induced minicells (8) containing the cloned *Eco*RI-H fragment of plasmid NR1.

There is a gap in our understanding of the *mer* operon that goes back to our original work on organomercurial resistance conferred by plasmid NR1 (30). NR1 confers resistance to Hg^{2+}, merbromin, and fluoresceinmercuric acetate; Weiss (A. A. Weiss, unpublished data) demonstrated that merbromin and fluoresceinmercuric acetate resistance depends upon prior induction by low levels of Hg^{2+} or organomercurials. Yet mercury is volatilized only from Hg^{2+} and not from these organomercurials (29, 30). The obvious choice for a nondegradative mechanism of

resistance would be a permeability barrier, perhaps specified by still another gene in the *mer* operon. A permeability block governed by an additional *mer* gene or as a pleotrophic attribute for *merT* is difficult to visualize. It must function in the direction opposite (i.e., to lessen uptake) to the *merT*-stimulated uptake of Hg^{2+}. Furthermore, this resistance is missing in plasmid pRR134 and in the Tn*A* mutants of the mercuric reductase gene (11). Attempts to measure binding of the organomercurials directly showed that both sensitive and hypersensitive cells bound less than 1% of the organomercurials and showed no differences in this regard from the resistant strain.

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