Interaction of Virion Protein Vpr of Human Immunodeficiency Virus Type 1 with Cellular Transcription Factor Sp1 and trans-Activation of Viral Long Terminal Repeat

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Acquired immunodeficiency syndrome (AIDS) is a result of replication of the human immunodeficiency virus type 1 (HIV-1) predominantly in CD4+ T lymphocytes and macrophages. However, most of these cells in vivo are immunologically quiescent, a condition restricting HIV-1 replication. Vpr is an HIV-1 virion protein suspected to enhance HIV-1 replication in vivo. We demonstrate in this report that Vpr specifically activates HIV-1 long terminal repeat (LTR)-directed transcription. This effect is most pronounced on a minimal promoter from HIV-1 LTR containing the TATA box and binding motifs for the ubiquitous cellular transcription factor Sp1. Evidence is presented that Vpr interacts with Sp1 when Sp1 is bound to the Sp1 motifs within the HIV-1 LTR. Both Vpr-Sp1 interaction and Vpr trans-activation require a central Leu/Ile-rich domain in Vpr. Our findings suggest that Vpr trans-activation through Sp1 is most critical for the immediate early transcription of HIV-1 when other positive regulators, such as NF-kB, are limited or inactive, a condition presumably present in vivo. By interacting with Sp1, Vpr also has the potential to influence cellular gene expression and cellular functions. Thus, therapeutic approaches directed toward blocking the Vpr trans-activation function could prove valuable in treating AIDS.

HIV-1 is the etiological agent of AIDS. The hallmark of AIDS is the slow but progressive depletion of CD4+ T cells, a class of T cells crucial for immune functions. Depletion of CD4+ T cell results in immunodeficiency and AIDS-related disorders, including encephalopathy, dementia, and malignancies (1). Despite tremendous efforts in the past, the mechanism of these AIDS-related disorders has remained unclear. However, it is clear that these are a consequence of function of HIV-1 encoded gene products. For example, the HIV-1 envelope glycoprotein was implicated to be involved in toxic effects on neuronal cells (2). Recently, the HIV-1 Vpr protein in peripheral blood of HIV-1-infected people was shown to activate HIV-1 replication in latently infected cells (3, 4). This effect of Vpr was suggested to contribute to HIV-1 pathogenesis in vivo.

The HIV-1 genome encodes structural as well as regulatory gene products (5, 6). Recently, great efforts have been made toward understanding the function of the so-called accessory regulatory genes, namely vif, vpr, vpu, and nef. These genes are generally non-essential for HIV-1 to replicate in activated T cells. Yet, animal model studies with two of these genes, vpr and nef, suggested that they are required for in vivo replication and pathogenesis of the simian immunodeficiency virus (7, 8). The paradox between HIV-1 replication in vitro and that in vivo suggests that HIV-1 replication may be subjected to different modes of regulation in vivo compared to in vitro. For example, in vitro studies have shown that HIV-1 replication is highly dependent on cellular activation and availability of activated NF-κB transcription factor (5, 6, 9). However, in vivo, the majority of susceptible cells are immunologically quiescent and do not have a high level of NF-κB activity to support a productive HIV-1 replication. Nevertheless, HIV-1 replication in vivo has been demonstrated to be relatively rapid (10).

To understand the role of HIV-1 accessory regulatory genes during HIV-1 replication and pathogenesis, we focused on the vpr gene product, which is a 96-aa protein produced late in the virus life cycle and assembled into the virion through binding to Gag (11–13). Function of Vpr appears to be critical for HIV-1 to replicate in macrophages (14). In lymphocytes, the effect of Vpr on HIV-1 replication is difficult to detect (15). Our earlier studies and results from others showed that Vpr has a tendency to localize in the nucleus (16, 17) without utilizing a classical nuclear localization signal (16). These results are consistent with the notion that Vpr may play a role during the nuclear migration of the pre-integration complex (18). However, they were also consistent with the hypothesis that Vpr can function in the nucleus to trans-activate HIV-1 (19). We report here that Vpr trans-activates HIV-1 LTR through interaction with the cellular transcription factor Sp1. Sp1 is an O-glycosylated transcription factor which binds to decanucleotide Sp1 motifs (consensus core sequence: GGCGCG) through three zinc finger domains (20–22). It is ubiquitously expressed and is involved in transcription of a variety of cellular genes including the proto-oncogenes Ha-ras-1 (23) and pim-1 (24). Purified Sp1 protein was shown to bind to all three Sp1 motifs within the HIV-1 LTR (25). We found that Vpr trans-activation was more dramatic when the transcriptional activity of HIV-1 LTR was lower. Our results are consistent with the notion that Vpr function is most critical for the immediate early transcription of HIV-1 when other positive regulators, such as NF-κB, are limited or inactive, a condition presumably present in vivo.
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**MATERIALS AND METHODS**

Plasmid Constructs and Transcription Templates—The EcoRI/HindIII fragment of the plasmid pU3RCATIII,2 containing the HIV-1 LTR plus 500-base pair flanking sequence, was cloned into pUC18 vector to generate pUC-HIV-1-LTR, which was digested with EcoRI/NdeI and used as the LTR transcription template (Fig. 1A). To obtain the NF-κB-Sp1 template (Fig. 1A), the pUC-HIV-1-LTR was digested with ScaI (immediately upstream of the NF-κB motifs) and PvuII (in the pUC18 vector). The three Sp1 motifs together with the five 82-base pair transcribed region of the HIV-1 LTR was amplified by polymerase chain reaction and cloned the same way as pUC-Sp1-LTR to generate pUC-TATA-LTR, which was digested with EcoRI/PvuII to generate the Sp1 template. Sequence from the TATA box to position +18 was amplified by polymerase chain reaction and cloned in the same way as pUC-Sp1-LTR to generate pUC-TATA-LTR, which was digested with EcoRI/PvuII to serve as the TATA template. Plasmid pDN-AdML (26) containing the TATA box basal promoter region of the adenovirus major late promoter was digested with EcoRI and NdeI to generate a 270-base transcribed.

The p3×Sp1-CAT plasmid was obtained by insertion of the EcoRI/HindIII region of pUC-Sp1-CAT plasmid into a vector containing the CAT reporter gene. The vpr(wt) gene in pET-vpr(wt) was transferred to the RSV-HndpFFH plasmid (16) to generate RSV-vpr(wt) plasmid. The construction of RSV-vpr-L-mu and RSV-S-vpr-T has been described (16).

Cell Culture, Preparation of Nuclear Extracts, and Transfection—HeLa cells were grown in suspension medium (J oklik’s modified minimal essential medium, Life Technologies Inc.) supplemented with 5% horse serum. CEM ×174 and U937 cells were cultured in RPMI medium supplemented with 10 and 20% fetal bovine serum, respectively. Nuclear extracts were prepared from cell lines according to established procedures (32) except KCl was used to replace NaCl. CEM ×174 cells were transfected with 3 μg of the p3×Sp1-CAT plasmid in the presence or absence of activator plasmids by using the DEAE-dextran method, and CAT assay was performed at 48 h post-transfection with a reaction time of 8 h following established protocols (29).

In Vitro Transcription Reaction—In vitro transcription reaction was carried out as described (22) using 1 μg of template DNA and radiolabeled UTP. All Vpr related proteins were purified as described before (16). Transcription reaction was carried out in a 50-μl volume with 20 μl of the crude nuclear extract (approximately 200 μg of total protein) at 30 °C for 45 min. The reaction was stopped with 50 μl of a buffer containing 0.6 M sodium acetate, 2 mM magnesium, 20 mM EDTA, and then extracted with phenol twice and precipitated with ethanol. The precipitated RNA was dissolved in a formamide loading buffer, electrophoresed on a sequencing gel, and transcription activity was quantitated with the help of a Phosphorimager (Molecular Dynamics).

**RESULTS**

In Vitro Transcriptional Activation of HIV-1 LTR by Vpr through Sp1 Binding Motifs—The nuclear localization of Vpr and its presence in the virion is consistent with the notion that Vpr functions in the nucleus to trans-activate immediate early transcription of HIV-1. To examine this possibility, we performed in vitro transcription experiments with the HIV-1 LTR template and purified Vpr-T which carries a C-terminal tag (14) (Fig. 1A). The LTR template was prepared by digestion of the plasmid pUC-HIV-1-LTR with restriction enzymes such that a 293-base run-off transcript could be generated. With nuclear extracts prepared from HeLa, CEM ×174, and U937 cells, Vpr-T increased HIV-1 LTR-directed transcription by more than 100% (Fig. 2A). The transcript was RNA-polymerase II derived since it was specifically inhibited by α-amanitin (data not shown). In addition, as described next, progressive depletion of HIV-1 LTR also progressively reduced this transcriptional activity.

The HIV-1 LTR contains as positive regulatory elements two binding motifs for the transcription factor NF-κB followed by three binding motifs for the Sp1 transcription factor (5, 6). To decipher which region of the HIV-1 LTR mediated response to Vpr-T, three templates containing: (a) NF-κB and Sp1 motifs (NF-κB-Sp1 template), (b) Sp1 motifs (Sp1 template), or (c) neither NF-κB nor Sp1 motifs (TATA template), were prepared (Fig. 1A). These templates produced a shorter mRNA transcript than the full-length LTR template, and were individually mixed with the LTR template in equal molar amounts for in vitro transcription with different amounts of Vpr-T. It was clear that the NF-κB-Sp1 template and the Sp1 template were both responsive to Vpr-T (Fig. 2, A and D) while the TATA template had no detectable basal activity and no Vpr response (data not shown). Among the three templates shown (Fig. 1A), the Sp1 template had the lowest level of basal activity (Fig. 2C, lane 1). However, it was activated by Vpr the most: the maximum gain in transcription reached 400% (Fig. 2D). Thus, it

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**Fig. 2. In vitro trans-activation of the HIV-1 LTR by Vpr.** A, in vitro transcription with nuclear extracts prepared from different cell lines. Where indicated, HIV-1 Vpr-T protein (16) (0.5 μg) was included in the transcription reaction. M, 1-kilobase DNA ladder (Life Technologies, Inc.) end-labeled with 32P. B, quantitation of transcripts by Phosphoimager (Molecular Dynamics, Inc.). Values were graphed as when the TATA box, at position 40 of the HIV-1 LTR (refer to Fig. 1), the resulting template did not respond to Vpr (data not shown).

Control experiments were carried out with two other Vpr-related proteins: the authentic Vpr (Vpr(wt)), and the Vpr-T mutant: Vpr-LR-mu which contains a mutation of 8 aa residues in the Leu/Ile-rich domain (LR-domain, aa numbers 60–81) of Vpr (16). For this experiment, the Sp1 template was used alone. It was clear that the authentic Vpr(wt) activated transcription of the Sp1 template to the same extent as Vpr-T while the ability of the Vpr-LR-mu protein to trans-activate was impaired (Fig. 3, A and B). We noticed that under this condition the maximum Vpr trans-activation was lower than when the Sp1 template and the LTR template were used together (Fig. 2C, lower panel). Thus, it seemed that the LTR template competed for the basal transcription machinery much more efficiently than the Sp1 template when Vpr was absent. However, when Vpr was present, the Sp1 template gained a competitive advantage.

A template containing the TATA box basal promoter of adenovirus major late promoter (AdML) was also mixed with the Sp1 template in equal molar amounts and tested for potential activation by Vpr-T. As Fig. 3C shows, the Sp1 template gave a much higher level response to Vpr-T than the AdML template, although the latter template was also significantly activated by Vpr-T. Inspection of the AdML sequence revealed two blocks of GC-rich sequences surrounding the TATA box (26). Preliminary studies suggested that Sp1 could bind to these sequences (data not shown). Thus, it may be possible that the observed activation of AdML promoter by Vpr also involved Sp1 binding to AdML. This may be in contrast to an earlier report which showed that Sp1 significantly activated a promoter in the absence of an identifiable Sp1 motif (27).

**Fig. 3. Specificity of Vpr trans-activation.** A, transcription reaction was carried out with 0.25, 0.5, and 1 μg of Vpr-T (lanes 2–4), Vpr(wt) (lanes 5–7), and Vpr-LR-mu (lanes 8–10) using 1 μg of the Sp1 template. B, the highest activation indices observed for the three Vpr proteins were plotted for comparison. C, transcription reaction with 0.5 μg each of the Sp1 template and the AdML template. D, activation indices for both templates at different Vpr-T concentrations were plotted for comparison.

**Transcription Factor Sp1**—Sp1 is an ubiquitous transcription factor which binds to decanucleotide Sp1 motifs (consensus core sequence: GGCGCGG) through three zinc finger domains (20–22). It is ubiquitously expressed and is involved in transcription of a variety of cellular genes including the proto-oncogenes Ha-ras-1 (23) and pim-1 (24). Purified Sp1 protein was shown to bind to all three Sp1 motifs within the HIV-1 LTR (25). These Sp1 motifs were synthesized as a double-stranded oligonucleotide (3×Sp1, Fig. 1B), radiolabeled, and used in a gel electrophoretic mobility shift assay with purified Sp1 protein and Vpr-T (Fig. 4A). Under the limiting concentration of purified Sp1 protein, Sp1 bound to the probe and generated a predominant Sp1-probe complex which was most likely due to Sp1 binding to one Sp1 motif in the probe.
In addition, a weak band corresponding to Sp1 binding to two motifs was also visible. The Sp1-probe complex was recognized by a monoclonal Sp1 antibody (lane 7). Interestingly, inclusion of Vpr-T also caused a mobility shift of the Sp1-probe complex (lane 2). Since Vpr-T itself did not bind to DNA (lane 8), it seemed possible that Vpr-T physically incorporated into the Sp1-probe complex. Alternatively, Vpr-T induced Sp1 binding to more Sp1 motifs in the probe. Incubation of the Vpr-T-induced complex with the Flag IgG-Affi-Gel for the tag in Vpr-T (16) depleted this complex (data not shown), suggesting that the first possibility was correct. This interpretation was also supported by the co-precipitation results (see next).

In the same assay, the authentic Vpr(wt) protein also induced a mobility shift of the Sp1-probe complex (Fig. 4A, lane 5). The apparently higher efficiency of Vpr(wt) was most likely due to the higher amount of Vpr(wt) used as compared to Vpr-T, since doubling the amount of Vpr-T also generated the same pattern. The Vpr-LR-mu protein, which failed to trans-activate the Sp1 template (Fig. 3A), had a severely reduced activity (lane 3) and VprΔ43-96 had no significant activity (lane 4). Interestingly, the SVmac Vpr protein (S-Vpr-T) expressed and purified the same way as HIV-1 Vpr-T (16), was not active (lane 6), suggesting that there is a host specificity in the Sp1 protein or a viral specificity in the Vpr protein. The potential existence of host specificity in Sp1 was consistent with the observation that with a HeLa nuclear extract S-Vpr-T did not trans-activate either HIV-1 LTR or SVmac LTR, while HIV-1 Vpr trans-activated both templates (data not shown). With a control system, the ATF-1 (29) bound to the cyclic AMP-responsive element (Fig. 4A, lane 15), but was not affected by Vpr-T (lane 16).

The Vpr-Sp1 interaction was further examined by co-immunoprecipitation assays. First, the DNA-protein complex formed as per the gel shift assay was co-precipitated by the Flag IgG-Affi-Gel which recognizes the C-terminal tag in Vpr-T (16). The precipitated DNA probe was examined by gel analysis and autoradiography. It was clear that the probe was co-precipitated only with Vpr-T (Fig. 4B, lane 3), but not with Vpr-LR-mu (lane 4) or S-Vpr-T (lane 5). Second, the Sp1 protein and Vpr-T protein were directly mixed and then co-precipitated with the Flag IgG-Affi-Gel. The precipitated proteins were examined for the presence of Vpr-T and Sp1 proteins by Western blot. Under this condition, Sp1 and Vpr-T did not co-precipitate (data not shown). However, when during the co-precipitation an excess of unlabeled 3×Sp1 oligo was included, Sp1-Vpr-T co-precipitation was observed (Fig. 4C, lane 3). Under this condition, Sp1 did not co-precipitate with Vpr-LR-mu (lane 4) or S-Vpr-T (lane 5). The above results are consistent with the conclusion that Vpr interacts with Sp1 in the context of the Sp1-DNA complex. To decipher whether individual Sp1 motifs can support Vpr-Sp1 interaction, the three Sp1 motifs in HIV-1 LTR were synthesized separately, and used for the gel mobility shift assay (Fig. 1B). None of these single Sp1 motifs supported Vpr-Sp1 interaction efficiently (Fig. 4A, lanes 9–14). In addition, during the co-precipitation analysis, the radiolabeled Sp1(I) probe was not co-precipitated with Vpr-T and Sp1 (Fig. 4B, lane 3). Thus, the dependence of Vpr-Sp1 interaction on the 3×Sp1 DNA, as shown by both the gel mobility shift assay and the co-precipitation assays (Fig. 4), suggests that Vpr forms a stable complex with Sp1 only when Sp1 binds to multiple Sp1 motifs in the 3×Sp1 DNA.

In Vivo Vpr trans-Activation through Sp1 Motifs—To examine if Vpr can trans-activate through Sp1 in vivo, we performed co-transfection experiments followed by CAT assay. With the

**Fig. 4. Vpr-Sp1 interaction.** A, gel mobility shift assay with Sp1 protein (25 ng, Promega) for lanes 1–7 and 9–14, and ATF-1 protein (25 ng) for lanes 15 and 16. Sp1 Ab is a mouse monoclonal for Sp1 (1 µg, Santa Cruz Biotechnology). The assay (10 µl) contained 0.1 ng of labeled probe and 5 ng of poly(dI·polydC) under a condition as described earlier. Human Sp1 protein was purified from HeLa cells by Promega following an established protocol (20). The probe sequences are listed in Fig. 1B. B, co-precipitation of the 3×Sp1 probe, but not the Sp1(I) probe, with Sp1 and Vpr-T by the Flag antibody Affi-Gel (IBI) that recognizes the C-terminal tag in Vpr-T. Lane 1 shows 20% of the input probe mixture without precipitation. Radioactive bands in between the two probes and below the Sp1(I) probe are single-stranded oligos. C, co-precipitation as in B except unlabeled 3×Sp1 oligo (100 ng) was used and the precipitated proteins examined by Western blot with Sp1 antibody and the Flag antibody. Lane 1 was 100% of the input proteins directly examined.

CAT reporter plasmid pU3CATIII containing the full-length HIV-1 LTR, and the RSV-vpr-wt plasmid expressing authentic Vpr, we observed about 2–3-fold trans-activation by Vpr in transfected cells (data not shown), consistent with the earlier report (19). To examine if Vpr can trans-activate through the Sp1 motifs alone, the three Sp1 motifs plus the TATA box of the HIV-1 LTR were cloned upstream of the CAT gene to obtain the p3×Sp1-CAT construct. As shown in Fig. 5, although the basal CAT activity was low (lane 1), maximum trans-activation by Vpr reached 7-fold (lane 2). In the same analysis, RSV-vpr-LR-mu (lane 5) and RSV-S-vpr-T (lane 6) did not trans-activate significantly. These results were consistent with the finding that Vpr-LR-mu and S-Vpr-T did not interact with human Sp1 (Fig. 4). We noticed that trans-activation by Vpr plateaued at a relatively low concentration of the Vpr-expressing plasmid. This is consistent with the results from Courey et al. (27) that trans-activation by Sp1 during co-transfection and CAT assay plateaued at a low concentration of the Sp1-expressing plasmid. The lower level of trans-activation by higher levels of RSV-vpr-wt may be accounted for by the cytotoxic effects of Vpr (30) which could be a result of abnormal regulation of cellular genes by Vpr interaction with Sp1.

**DISCUSSION**

In this report, we identified the ubiquitous cellular transcription factor Sp1 to be a target for HIV-1 Vpr. Vpr interaction with Sp1 was correlated with Vpr trans-activation of HIV-1 LTR. Maximum in vitro trans-activation was found with a minimal promoter from HIV-1 LTR that contains only three
Sp1 motifs and the TATA box. This minimal promoter also had the lowest detectable level of basal transcription activity among the templates tested. It is likely that trans-activation of the HIV-1 LTR by Vpr plays an important role for the immediate early transcription of the HIV-1 genome when the alternative positive regulations, such as the one committed by NF-kB, are low. This condition apparently exists in vivo where the majority of HIV-1 infected cells are immunologically quiescent.

Vpr interaction with Sp1 and trans-activation both required the central Leu/Ile-rich domain (LR-domain, aa numbers 60–81), which was previously reported to be important for Vpr interaction with a 180-kDa cellular protein RIP (16). However, Vpr trans-activation through Sp1 apparently is an event separable from Vpr interaction with RIP, since a point mutation of Arg62-Ser within the LR-domain was found to abolish Vpr-RIP interaction but not Vpr interaction with Sp1 or Vpr trans-activation.3 It may be proposed that RIP does not participate directly in the trans-activation process, but rather regulates Vpr stability or participates in Vpr nuclear migration as previously suggested (16).

Vpr-Sp1 interaction appeared to require Sp1 binding to multiple Sp1 motifs within the HIV-1 LTR. Individual Sp1 motifs did not support Vpr-Sp1 interaction efficiently (Fig. 4). We have recently obtained data that the two Sp1 motifs distal to the TATA box in HIV-1 LTR are sufficient to support Vpr-Sp1 interaction (data not shown). Based on these observations, a model may be proposed for Vpr-Sp1 interaction (Fig. 6). In pathway a, Sp1 monomer binds to the two Sp1 motifs in the DNA and forms an unstable dimer. This DNA-Sp1 dimer complex may dissociate during the gel mobility shift assay, leaving predominantly a complex containing a monomeric Sp1 (Fig. 4A). However, Vpr probably interacts with the dimeric Sp1 and stabilizes Vpr-Sp1 monomer-DNA complex. In addition, monomeric Sp1 bound to a single Sp1 motif may also interact with Vpr (Fig. 6, top), resulting in a Vpr-Sp1 monomer-DNA complex. This complex is presumably unstable, but can be strengthened by additional binding by another Sp1 monomer to the second Sp1 motif. Since Vpr exists as an oligomer (31), it should be possible that one Vpr oligomer can bind to two adjacent Sp1 molecules. We noticed that each of the three Sp1 motifs in HIV-1 LTR occupies one α-helical turn (10 base pairs) with 1 base pair space between adjacent Sp1 motifs. Thus, two adjacently bound Sp1 molecules are most likely on one side of the α-helical DNA. This alignment is expected to be best for simultaneous interaction of two adjacent Sp1 molecules with the Vpr oligomer. In pathway b, Vpr directly interacts with free monomeric Sp1, and forms a Vpr-Sp1 monomer complex. This complex is unstable, and additional weak interaction between the Vpr-Sp1 monomer complex with another Sp1 monomer does not help stabilize this complex.

Our data also suggested that the Vpr proteins of HIV-1 and SIVmac may be involved in host- or viral-specific protein-protein interaction and trans-activation. The SIVmac Vpr does not interact with the human Sp1 protein (Fig. 4) or trans-activate with the HeLa nuclear extract (data not shown). Although the C-terminal region of Vpr is most divergent between SIVmac Vpr and HIV-1 Vpr, preliminary analysis suggested that this region is not essential for Vpr-Sp1 interaction or Vpr trans-activation (data not shown). Instead, our current study identified the LR-domain to be important for Vpr-Sp1 interaction and Vpr trans-activation. Comparison between the LR-domains of HIV-1 Vpr and SIVmac Vpr revealed that there are five amino acid differences within the 22-aa LR-domain (16). When the LR-domain of SIVmac Vpr was substituted for that of HIV-1 Vpr in the context of an infectious HIV-1 clone, a chimeric HIV-1 virus was obtained that showed a delayed replication kinetics in CEM×174 cells compared with the wild type HIV-1 virus.3 This observation is consistent with the possibility that the LR-domain is involved in host- or viral-specific functions of HIV-1 and SIVmac Vpr proteins. It also implied that Vpr trans-activation involving the LR-domain confers an advantage to HIV-1 replication even in lymphocytes.

In summary, we provided biochemical evidence to support a specific trans-activation function associated with the HIV-1 Vpr. By targeting a ubiquitous cellular transcription factor Sp1, Vpr could affect not only HIV-1 replication, but also expression of cellular genes. Both effects of Vpr could contribute to AIDS pathogenesis. Thus, therapeutic approaches directed against the trans-activation function of Vpr should have two advantages: arresting HIV-1 replication at the very early stage of the virus life cycle, and relieving symptoms potentially caused by altered cellular gene expression induced by Vpr.

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