Regulation of Transforming Growth Factor-β1 Expression by the Hepatitis B Virus (HBV) X Transactivator

Role in HBV Pathogenesis

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Abstract

TGF-β1 has been implicated in the pathogenesis of liver disease. The high frequency of detection of the hepatitis B virus X (HBx) antigen in liver cells from patients with chronic hepatitis, cirrhosis, and liver cancer suggested that expression of HBx and TGF-β1 may be associated. To test this possibility, we examined the expression of TGF-β1 in the liver of transgenic mice expressing the HBx gene. We show that the patterns of expression of TGF-β1 and HBx protein are similar in these mice and that HBx activates transcription of the TGF-β1 gene in transfected hepatoma cells. The cis-acting element within the TGF-β1 gene that is responsive to regulation by HBx is the binding site for the Egr family of transcription factors. We further show that the Egr-1 protein associates with the HBx protein, allowing HBx to participate in the transcriptional regulation of immediate–early genes. Our results suggest that expression of HBx might induce expression of TGF-β1 in the early stages of infection and raise the possibility that TGF-β1 may play a role in hepatitis B virus pathogenesis. (J. Clin. Invest. 1996. 97:388–395.) Key words: hepatitis B virus • TGF-β1 • transcription • immunohistochemistry • hepatocellular carcinoma

Introduction

Hepatitis B virus (HBV)1 causes acute and chronic liver cell injury and inflammation and is strongly associated with liver cancer (1). Its genome contains four recognized open reading frames, three of which code for known virion proteins (2). The fourth, called the HBV X gene (HBx), is conserved among all mammalian hepadnaviruses and has been shown to be expressed both during viral infection (3–5) and in HBV-associated hepatocellular carcinoma (6). Recently, it has been shown that the woodchuck hepatitis virus X gene is important for the establishment of viral infection in woodchucks, suggesting that the HBx gene plays an important role in HBV replication in humans (7).

It is known that HBx trans-activates the expression of many viral and cellular transcriptional promoters (8–13). It activates transcription through certain cis-acting sequences, including transcription activator proteins AP-1 and AP-2 (14). HBx also forms protein–protein complexes with cellular transcription factors such as cyclic AMP-responsive element-binding protein (CREB) and activating transcription factor (ATF-2) and modifies their ability to bind transcriptional enhancers (15). Recently, it has been reported that HBx can activate protein kinase C, a key component of cellular signal transduction (16). Moreover, we have demonstrated that expression of the HBx gene alone is sufficient for the development of liver cancer in transgenic mice (17, 18).

Cytokines affect many functions in the liver, including amino acid, protein, lipid, mineral, and carbohydrate metabolism. In liver disease, cytokines are involved in the onset of intrahepatic immune responses, in liver regeneration, and in the fibrotic and cirrhotic transformation of the liver after chronic chemical injury or viral infection (19). To understand the mechanisms by which HBx induces changes in the liver, we examined the expression of TGF-β1, a cytokine that inhibits hepatocyte proliferation during liver regeneration (20–22) and stimulates the production of extracellular matrix proteins by hepatocytes during liver cirrhosis (23, 24). Recent results also suggest that TGF-β1 may play a role in the pathogenesis of fibrosis in chronic hepatitis and cirrhosis (25) and in the development of hepatocellular carcinoma (26).

In this study, we examined the expression of TGF-β1 in the livers of transgenic animals harboring the HBx gene under its own control elements (17, 18). TGF-β1 expression correlated well with the expression of the HBx protein in the early focal lesions of altered hepatocytes, suggesting that HBx acted either directly or indirectly to increase TGF-β1 expression. We also showed that HBx trans-activates the TGF-β1 promoter through the Egr-1 binding sites. Additionally, TGF-β1 expression was sustained in the adenoma and carcinoma lesions, suggesting that it might play a role in the progressive stages of hepatocellular carcinoma.

Methods

Immunohistochemistry. The liver tissues were fixed in 10% formalin and embedded in paraffin. 5-μm sections were immunostained with either anti-HBx antiserum or anti–TGF-β1 antibodies, visualized by the avidin–biotin complex method (Vector Laboratories, Inc., Burlingame, CA), and counterstained with hematoxylin. TGF-β1 antibodies used for immunohistochemistry were raised in rabbits to the NH2-ter-

1. Abbreviations used in this paper: AP, activator protein; ATF, activating transcription factor; CAT, chloramphenicol acetyltransferase; CREB, cyclic AMP-responsive element-binding protein; GST, glutathione S-transferase; HBV, hepatitis B virus; HBx, HBV X gene.

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minal 1–30 amino acids of mature TGF-β1 (anti-CC and anti-LC) (27, 28). Anti-CC stained extracellular matrix associated TGF-β1, whereas anti-LC stained intracellular TGF-β1.

**RNA isolation and blotting.** Total RNAs from mouse tissues were extracted by acid guanidinium thiocyanate–phenol chloroform extraction. Total RNAs (10 μg each) were separated by electrophoresis through 1% agarose–formaldehyde gels and transferred to nitrocellulose membranes. Prehybridization, hybridization, and washing of the membrane were as described previously (29).

**Cell culture, DNA transfection, and chloramphenicol acetyltransferase (CAT) assays.** HepG2 cells were grown in Dulbecco’s minimal essential medium supplemented with 10% FBS. For transient expression assays, cells were plated at 1.2 × 10^6 per 10-cm dish and cultured for 24 h before transfection by the calcium phosphate coprecipitation method. Cells were harvested 48 h after addition of DNA, and extracts were assayed for CAT activity. All transfections were repeated at least three times. For normalization of transfection efficiencies in HepG2 cells, a growth hormone expression plasmid (pSVGH) was included in the cotransfections. The level of growth hormone expression was determined using a growth hormone detection kit (Nichols Institute, San Juan, Capistrano, CA).

**Mobility shift assays.** Bacterially expressed Egr-1 protein was kindly provided by Dr. V. Sukhatme (30). The double-stranded oligonucleotides were labeled by Klenow enzyme and gel purified. After bacterially expressed Egr-1 protein was incubated for 25 min at room temperature in binding buffer (20 mM Hepes, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 2 μg of double-stranded poly[dI-dC] nonspecific competitor, ~ 0.2 ng of 32P-labeled probe), the reaction products were loaded onto a 5% polyacrylamide gel (39:1, acrylamide/bisacrylamide) and electrophoresed in 0.5 × TBE (50 mM Tris, 50 mM boric acid, 1 mM EDTA) for 2.5–3.5 h at 8 V/cm. Gels were dried and autoradiographed.

**Plasmids.** Human TGF-β1 promoter/CAT plasmids (29), GAL4-ATF-2 (31), GAL4-CREB (32), and G5BCAT reporter constructs (33) have been previously described. All GAL4-Egr-1 fusion plasmids were constructed by inserting the appropriate Egr-1 DNA fragment in-frame to the GAL(1–147) sequence in the vector pSG424 (34). Egr-1 DNA fragments were produced by polymerase chain reaction. The 5′-oligonucleotide used in all amplifications contained an EcoRI site and the 3′-oligonucleotide contained an XbaI site. Using these oligonucleotides, fragments were amplified according to the standard protocol of the GeneAmp kit (Perkin-Elmer Corp., Norwalk, CT). The junctions of all GAL4 fusion plasmids were confirmed by DNA sequencing. The HBx expression plasmids (pMAM

**Figure 1.** Multifocal areas of altered hepatocytes and benign tumor nodules in the liver of HBx-transgenic mice. Serial sections of a liver from a 4-mo-old male transgenic mouse were stained with hematoxylin and cosin (A), immunostained with a rabbit anti-HBs serum against a synthetic peptide spanning residues 100–115 (B), or immuno-stained with an antipeptide antibody raised to amino acids 1–30 of TGF-β1 (LC 1-30-1) that detects intracellular TGF-β1 (C). Age-matched nontransgenic mouse liver was immunostained with anti-TGF-β1 antibody against intracellular TGF-β1 as a control (D). Serial sections of liver with an adenoma from a 15-mo-old male transgenic mouse were stained with hematoxylin and cosin (E), immunostained with the anti-HBs serum (F), immunostained with anti-TGF-β1 antibody (LC 1-30-1) for intracellular TGF-β1 (G), or stained with anti-TGF-β1 antibody (CC 1-30-1) for extracellular TGF-β1 (H).
ND1-4 and CD1-4) used for the expression of native and mutant HBx proteins in eukaryotic cells were constructed by cloning the HBx-ORF insert into the SalI site of pMAM-neo (Promega, Madison, WI).

Glutathione S-transferase (GST)-HBx and GST-Egr-1 fusion proteins. Various GST-HBx and eight GST-Egr-1 fusion proteins expressed in Escherichia coli were partially purified by adsorption to glutathione-Sepharose beads (Pharmacia Biotech Inc., Piscataway, NJ) in the presence of the detergent N-laurylsarcosine and Triton X-100 as has been described previously (35).

Egr-1 proteins and HBx protein generated by in vitro transcription and translation. Plasmids containing the Egr-1 (1–533), Egr-1 (32–533), Egr-1 (147–533), Egr-1 (249–533), and Egr-1 (1–364) were constructed by inserting appropriate PCR-generated fragments of Egr-1 at in-frame EcoRI/BamHI restriction sites in pGEM4 (Promega). For the synthesis of [35S]methionine-labeled HBx protein by in vitro transcription and translation, pTM1/HBx plasmid was used as template for RNA synthesis by T7 RNA polymerase followed by translation in rabbit reticulocyte extracts (Promega).

Results

Expression of TGF-β1 in transgenic mice harboring the HBx gene. At 4 mo of age, the HBx-transgenic mice exhibited multifocal areas of altered hepatocytes that were made up of cells with a poorly stained cytoplasm (Fig. 1A) and were not detected in nontransgenic littermates (17, 18). Expression of the gene.

At 4 mo of age, the HBx-transgenic mice exhibited multifocal areas of altered hepatocytes that were made up of cells with a poorly stained cytoplasm (Fig. 1A) and were not detected in nontransgenic littermates (17, 18). Expression of the HBx protein as seen by immunohistochemical staining of a serial liver section using an anti-HBx serum correlated precisely with the altered foci (Fig. 1B), consistent with its role in the underlying histopathological change. The restricted expression of HBx in only a subset of cells may reflect the need for specific transcription factors required for the activation of the viral regulatory elements and present only in cells at a specific differentiation state.

Expression of TGF-β1 was assessed by immunohistochemical staining using isotype-specific peptide antibodies. While TGF-β1 was not detected in the liver of normal mice (Fig. 1D), it was found in the livers of the transgenic mice (Fig. 1C). Interestingly, the cells that expressed TGF-β1 were located exclusively within the altered foci made up of hepatocytes (17) where HBx was also highly expressed (compare Fig. 1, B and C), supporting the possibility that TGF-β1 expression could be activated by HBx. Whereas every cell within an altered focus expressed HBx, only a subset of them also expressed TGF-β1. This latter observation suggests that HBx expression, while necessary, is insufficient to induce TGF-β1 and argues for epigenetic rather than genetic events underlying TGF-β1 activation by HBx.

At ~8–12 mo of age, tumor nodules begin to appear in the livers of the transgenic mice. While many of these tumors had benign characteristics and were diagnosed as adenomas (Fig. 1E), others appeared malignant and resembled hepatocellular carcinoma (Fig. 2). HBx protein was highly and uniformly expressed in both adenoma and carcinoma lesions (Figs. 1F and 2A, respectively). Similarly, TGF-β1 expression was also detected in the tumors. For adenomas, the accumulation of intracellular TGF-β1 appeared to differ between individual cells as has been observed for cells in the altered foci, suggesting that, while its expression is dependent on the HBx protein, other factors may also be involved (Fig. 1G). For carcinomas, the cells that expressed TGF-β1 were localized to discrete regions (Fig. 2B). Interestingly, extracellular TGF-β1 is detected only in malignant tumors (Fig. 2C and D) and not in benign lesions (Fig. 1H). However, we cannot exclude the possibility that this represents a relative rather than an absolute difference in the level of accumulation between the two stages of the disease process.

Northern blot hybridization analysis of RNA extracted from a control mouse liver and from a transgenic liver with focal lesions, adenomas, or carcinomas suggested that the increase in TGF-β1 expression in tumors may be regulated at the transcriptional level. The level of TGF-β1 mRNA was undetectable in the control liver, but expressed at high levels in the tumor (Fig. 3). Taken together, our results demonstrate a
strong association between expression of HBx and TGF-β1 in vivo and suggest the possibility that TGF-β1 might play a part in early stages of development of hepatocellular carcinoma induced by HBV infection.

Trans-activation of the TGF-β1 promoter by HBx is mediated through the Egr-1 binding sites. To investigate whether increased expression of TGF-β1 in liver from the HBx-transgenic mice was transcriptional, we examined the promoter activities of the TGF-β1 gene. The structures of the TGF-β1-CAT chimeric plasmids used in this study have been described previously (29, 36). The chimeric plasmids were cotransfected into the hepatocarcinoma cell line, HepG2, with the control expression plasmid (pMAM) or with this same plasmid engineered to express HBx (pMAM-HX). Expression of the TGF-β1-CAT reporter gene (pHTG7-1) was 27-fold higher with pMAM-HX than with pMAM (Fig. 4, compare lanes 1 and 2).

We next sought to identify the specific cis-acting element that mediates responsiveness to HBx by testing a series of deletion constructs of the TGF-β1 promoter linked to CAT. The induction dropped almost to the basal level when the deletion reached −71 (Fig. 4, compare lanes 7 and 8).

The TGF-β1 promoter contains two Egr-1 binding sites at positions −119 to −111 (5′-CCGCGCCCGC-3′) and −82 to −74 (5′-CCGGGGCCGC-3′) (Figs. 4 and 5). To determine if these sites are specifically involved in HBx-mediated transcriptional regulation, we generated chimeric constructs containing sequences between −125 and −98 and between −93 and −63 ligated to the adenovirus E4Δ-38 promoter-CAT vector (37). Three-base substitution mutants of the Egr-1 binding sites were also generated and tested for HBx transactivation (Fig. 5, A and B). No increase in CAT activity was observed when the control plasmid pE4Δ-38 was cotransfected with pMAX-HX, whereas pE4Δ-38/−125/−98 and pE4Δ-38/−93/−63 that contain the separate Egr-1 sites both showed an increase in CAT activity under the same conditions (Fig. 5, A and B, respectively). Mutant constructs, pE4Δ-38/−125/−98mt) and pE4Δ-38/−93/−63mt), were not activated by HBx (Fig. 5 A, lanes 5 and 6, and Fig. 5 B, lanes 3 and 4). We also demonstrated that bacterially expressed Egr-1 binds to these two Egr-1 binding sites (29, data not shown).

A GAL4-Egr-1 fusion protein can mediate transcription activation by HBx. To confirm that the Egr-1 protein was directly involved in HBx-mediated transcriptional activation of TGF-β1, we designed a protein fusion experiment. Plasmids expressing various GAL4 fusion proteins were cotransfected with a CAT reporter construct (G5E1bCAT), which contained five GAL4 binding sites upstream of the AdE1b TATA box (33). To these transfection mixtures we added either the HBx expression plasmid (pMAM-HX) or the control expression vector (pMAM). As expected, HBx did not stimulate transcription on cotransfection of the minimal GAL4 DNA-binding domain (Fig. 6, lanes 1 and 2). On cotransfection of GAL4-Egr-1, however, transcription was greatly stimulated by HBx (Fig. 6, lanes 7 and 8). Even though it has been shown that the HBx protein forms protein–protein complexes with both CREB and ATF-2 (15), transcriptional stimulation was observed with GAL4-ATF-2 (Fig. 6, lanes 9 and 10) but not with GAL4-CREB (Fig. 6, lanes 5 and 6). Transcriptional stimulation was also not observed on cotransfection of GAL4-ATF-1 (Fig. 6, lanes 3 and 4) or GAL4-VP1, an activator carrying an acidic activating region (Fig. 6, lanes 11 and 12). These results indicate that Egr-1...
can specifically support HBx-mediated transcriptional activation.

**Identification of the domain of HBx responsible for its trans-activation function.** The amino acid sequence of the HBx protein reveals relatively few structural motifs that might be involved in trans-activation. Several reports indicate that the COOH terminus may act as an acidic activator (38). We next examined the effect of HBx deletion constructs on GAL4-Egr-1 transcription to identify the active region of the HBx protein.

A series of NH2- or COOH-terminal deletion constructs was generated as shown in Fig. 7. The ND-4 construct, in which 50 amino acids were deleted from the NH2 terminus of the HBx protein, was still able to activate GAL4-Egr-1 transcription, whereas removal of up to 20 amino acids from the COOH terminus abolished the ability of the protein to activate transcription (Fig. 7). It had previously been demonstrated that the block of amino acids from position 132 to 139, containing the highly conserved sequence FVLGGCRH, is essential for maintaining the trans-activation function (39). Our results also suggest that the COOH terminus is required for activation of the TGF-β1 promoter.

The HBx protein binds to Egr-1 in vitro. We next investigated the ability of HBx to interact directly with Egr-1 in vitro. Fig. 8 shows the results of the GST affinity chromatography experiment. An intact 17-kD [35S]methionine-labeled HBx protein prepared by in vitro translation was found to bind the GST-Egr-1 protein by GST affinity chromatography (Fig. 8A, lane 2). The binding of HBx was not detected with agarose beads containing GST alone (data not shown). To identify which domain of Egr-1 binds to the HBx protein, seven GST-Egr-1 deletion chimeras, GST-Egr-1 (1–100), GST-Egr-1 (1–150), GST-Egr-1 (247–414), GST-Egr-1 (410–533), GST-Egr-1 (247–334), GST-Egr-1 (335–418), and GST-Egr-1 (427–534) were expressed and subjected to GST affinity chromatography. The results are shown in Fig. 8B.
(247–364), were constructed using internal PCR primers (Fig. 8 B). All proteins from these chimeras were produced from IPTG-induced E. coli bacterial hosts and analyzed by SDS-PAGE (data not shown). The GST affinity assay was used to show that HBx protein can bind to the GST-Egr-1. The results revealed that HBx could bind to intact Egr-1 (1–533), Egr-1 (247–414), and Egr-1 (335–418), all containing the three zinc finger domains (Fig. 8 A, lanes 2, 5, and 8), but not to Egr-1 (1–100), Egr-1 (1–150), Egr-1 (410–533), Egr-1 (247–334), and Egr-1 (247–364) (Fig. 8 A, lanes 3, 4, 6, 7, and 9).

The design of the GST bead affinity binding assay was reversed by using [35S]methionine-labeled in vitro–translated forms of Egr-1 to identify which segment of Egr-1 was involved in binding to HBx protein. In Fig. 9, a Sepharose bead–bound GST fusion protein containing the entire HBx protein was used. The results revealed that intact Egr-1 (1–533), Egr-1 (32–533), Egr-1 (147–533), and Egr-1 (249–533) could bind to the HBx protein. However, the NH2-terminal segment of Egr-1 (1–364), which does not contain the zinc finger domain, did not show any binding to HBx. [35S]Methionine-labeled in vitro–synthesized preparations of the intact human p53 and the ATF-2 protein served as positive controls, and the ATF-1 protein served as a negative control for the binding specificity in this assay (15, 40).

**Discussion**

It has been shown that HBx is capable of *trans*-activating a variety of viral (9, 12, 41) and cellular promoters (13, 16, 41). Recently, we also demonstrated that HBx is directly involved in the development of liver cancer by generating transgenic mice harboring the entire HBx gene (17). Using these same transgenic mice, we have now been able to show a strong association of expression of HBx and TGF-β1, which is known to play an important role in chronic hepatitis and liver cirrhosis (23–25) in vivo. Moreover, in vitro experiments demonstrate that HBx can directly induce the expression of TGF-β1. We demonstrated that TGF-β1 localized immunochemically to the HBx-expressing cells and that sequences homologous to a previously defined Egr-1–responsive element (30) mediate the regulation of TGF-β1 promoter activity by the HBx protein. These results suggest that TGF-β1 may be one of the mediators of HBV pathogenesis.

Egr-1, also known as TIS-8 (42), Zif268 (43–45), Krox 24 (46), and NGFI-A (47), is a recently characterized transcription factor that is responsive to cell division signals (48). It contains three zinc fingers, which bind to the target sequence GCGGGGCGG (48, 49), also recognized by the zinc finger transcription factor WT1 (50). Egr-1 is rapidly induced in response to a variety of stimuli (48). Like the protooncogenes c-fos and c-jun, Egr-1 is induced within 30 min by extracellular growth signals in the absence of protein synthesis and when cells of certain lineages are cued to differentiate. Promoters of many genes including insulin-like growth factor II (51) contain Egr-1 binding sites. These observations suggest that regulation of Egr-1 function may lead to changes of cell physiology.

Mutational analysis of HBx has demonstrated that multiple structural motifs are separately involved in the activation of different promoters (38). The COOH terminus contains the moderately acidic alpha helix. Removal of up to 12 amino acids from the COOH terminus did not change its *trans*-activating function in vitro (8, 39, 52–54), whereas deletion of amino acids between positions 132 and 139, containing the highly conserved sequence FVLLGGRH, resulted in the loss of activity (39). Interestingly, the HBx protein extends nine amino acids beyond that of the woodchuck hepatitis virus at the COOH terminus (7). It appears that the amino acids between positions 126 and 136 of woodchuck hepatitis virus are important for virus replication in the natural host, suggesting that this region is crucial to the function of the protein (7). In this study, we have also demonstrated that deletion of amino acids from positions 136 to 153 of the HBx protein abolished its *trans*-activating function.

It is known that the HBx protein does not bind directly to DNA but instead forms protein–protein complexes with cellular transcription factors like CREB and ATF-2, and alters their binding specificities (15). We have demonstrated that HBx stimulates transcription conferred by GAL4-ATF-2 but not by GAL4-CREB. Since ATF-2 lacks a constitutive activating region (31), a cellular factor like the retinoblastoma gene product (55) or a viral protein such as the adenovirus E1a may bind to ATF-2 and supply the transcriptional activating function. The fact that HBx is not able to induce transcription conferred by GAL4-CREB indicates that HBx only alters the DNA binding specificity of CREB and that the activation potential of CREB possibly requires phosphorylation (56, 57).

In the present study, we have demonstrated that HBx forms a complex with the Egr-1 protein. Because phosphorylation events modulate the activity of a number of transcription factors, this could impart another level of regulation on Egr-1 activity. It is of interest to note that Natoli et al. (58) have suggested that the HBx protein might induce posttranslational
modifications that render the API complex more efficient in its DNA-binding ability. Phosphorylation and dephosphorylation of c-Fos and c-Jun have been described and shown to have a critical role in the regulation of API function (59, 60).

TGF-β1 is an important cytokine in the pathophysiology of liver fibrosis, stimulating the production of extracellular matrix. In hepatic fibrosis, a marked increase is seen in the hepatic extracellular matrix proteins, including collagens, glycoproteins, and glycosaminoglycans. In both experimental models of hepatic fibrosis and in patients with liver cirrhosis, increased expression of type I collagen genes is seen (61). The TGF-βs also stimulate type I collagen gene expression in primary cultures of hepatocytes, Ito cells, and fibroblasts (23). Castilla et al. (25) have shown that the level of TGF-β1 mRNA in liver biopsy specimens correlated positively with hepatic fibrosis in a large group of patients with chronic viral hepatitis, suggesting that TGF-β1 may play a role in the pathogenesis of hepatic fibrosis.

TGF-β1 most often acts as a negative growth regulator (62). This effect must be overcome before tumor progression can occur (63). Evidence from animal models as well as human tumors indicates that malignant tumors including human hepatocellular carcinoma secrete high levels of TGF-β1 (26, 64–66). Increased secretion of TGF-β1 by cells that have lost responsiveness to its growth inhibitory activities is thought to facilitate tumor progression by indirect means such as suppression of immune surveillance and stimulation of tumor stroma. In this study, we have demonstrated that the level of TGF-β1 mRNA is significantly increased in livers from the HBx-transgenic mice and that expression of TGF-β1 correlates well with that of the HBx protein.

A recent study has shown that 84% of patients with primary hepatocellular carcinoma who are HBV carriers were HBx positive in their tumor cells (67). Evidence that expression of TGF-β1 correlates well with that of the HBx protein in transgenic mice and that TGF-β1 expression is also elevated in hepatic cirrhosis in patients with chronic viral hepatitis suggests that further work should be done to define the specific role of TGF-β1 in the pathogenesis of HBV-associated liver diseases, including primary hepatocellular carcinoma.

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References


