Negative Regulation of Hepatitis B Virus Gene Expression and Replication by Oxidative Stress*

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We present data demonstrating that hydrogen peroxide markedly decreases release of progeny hepatitis B virus particles in cultured cells. The presence of reduced glutathione prevents this effect. Hydrogen peroxide also decreases secretion of the hepatitis B virus surface and e antigens, with a concomitant decrease in the steady-state levels of the corresponding viral transcripts. This effect is specific to viral gene expression, since hydrogen peroxide at the concentration used does not have any significant effect on the overall pattern of host cell protein synthesis nor on the secretion of host cell protein. Since hydrogen peroxide at the concentration used does not have any significant effect on the overall pattern of host cell protein synthesis nor on the secretion of host cell protein, this phenomenon may be of pathophysiological importance in the viral life cycle.

Organisms must adapt to a variety of different environmental conditions to survive. This adaptation is frequently accomplished by a molecular sensor that detects an environmental parameter and a mechanism, either direct or indirect, to effect gene expression changes that enable the organism to thrive under those conditions. One environmental factor to which organisms must adapt is reactive oxygen species, which can be generated by external factors, such as UV radiation or molecules that undergo redox cycling, as well as by biological processes (principally aerobic metabolism) intrinsic to the organism (reviewed by Ahern (1991)). For example, in the bacterium Escherichia coli, the OxyR regulon is sensitive to hydrogen peroxide, by virtue of transcriptional activation of genes with upstream OxyR protein binding sites by oxidized but not by reduced OxyR protein (reviewed by Demple and Amabile (1991)). The products of these genes help to defend the bacterium by eliminating hydrogen peroxide and repairing damage to oxidized macromolecules.

Mammalian cells respond to oxidative compounds with changes in gene expression patterns, as well. Recent reports have shown that these changes may also be mediated by transcription factors that are sensitive to redox potential (Devary et al., 1991; Nosé et al., 1991; Schreck et al., 1991; Staal et al., 1990). For example, oxidative stress in the form of hydrogen peroxide activates latent NF-κB in intact cells (Schreck et al., 1991; Staal et al., 1990). Since phagocytic leucocytes release hydrogen peroxide and other reactive oxygen species in sites of inflammation (see reviews by Badwey and Karnovsky (1980); Cerutti and Trump (1991)), and since certain inflammatory cytokines may indirectly release intracellular oxidants (Cerutti and Trump, 1991), this phenomenon may be important in allowing NF-κB to activate defense mechanisms in the face of pathogenic agents. The human immunodeficiency virus (HIV) appears to have parasitized this system, allowing its transcription and replication to be activated when the latently infected host cell participates in the inflammatory response (Staal et al., 1990). It has been speculated that this subversion of host defense mechanisms may be one factor that causes immunodeficiency during HIV infection.

Another virus that can evade the immune system and cause chronic infections is the hepatitis B virus (HBV) (see reviews by Ganem and Varmus (1987); Hollinger (1990)). Since HBV infection frequently gives rise to inflammation in the liver with concomitant release of reactive oxygen species, we tested the effect of hydrogen peroxide as a physiological oxidant on HBV gene expression and replication in cultured cells. In contrast to HIV gene expression, HBV surface and e antigen (HBsAg and HBeAg, respectively) expression is markedly down-regulated by this treatment, with a corresponding decrease in the viral transcripts that code for the two antigens. There is a similarly marked decrease in the amount of progeny virion particles released without any significant difference in the overall pattern of host protein synthesis. This previously undescribed effect may be important in the pathophysiology of chronic hepatitis B virus infection.

EXPERIMENTAL PROCEDURES

Materials—Hydrogen peroxide (30%) and reduced glutathione (herein simply called glutathione) were purchased from Sigma. Hydrogen peroxide was freshly diluted before each use. The glutathione was dissolved in 1 x NaOH as a 1 x stock solution and the pH value adjusted to 7.4; the final concentration used was 15 mM. Enzymes were purchased from Boehringer Mannheim, Life Technologies, Inc., or New England Biolabs.

Plasmids and Cell Transfection—The plasmid pHBV2 contains a head-to-tail dimer of the HBV genome strain adw2 (Valenzuela et al., 1980) and is a kind gift of Jing-Hsiung Ou, University of Southern California.

The abbreviations used are: HIV, human immunodeficiency virus; HBV, hepatitis B virus; HBeAg, HBV e antigen; HBsAg, HBV surface antigen.

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RNA, Protein, DNA, and HBV Virion Analysis—Total cellular RNA was extracted with RNAzolB (Biotex), and 5 μg of the purified RNA was used for each primer extension reaction. Primer extension analysis of the HBV surface gene RNA was performed as previously described (Zhou and Yen, 1990). Primer extension of the core gene RNA was performed using the primer 5′GTTAAAGCACCATTGAGGACTCTAAGG (map positions 206–2024), which should give rise to products approximately 230 and 260 base pairs in length (Yaginuma and Koike, 1989). Primer extension of growth hormone RNA was performed using the primer 5′gGCCAcTCCAGCTAGGTGAGCGTCC; note that the two bases shown in lower case letters are nonhomologous to the RNA sequence, since the primer was originally designed for an amplification reaction. The expected size of the major extended product is 127 base pairs (Selden et al., 1986). The primer extension products were quantitated by a Molecular Dynamics PhosphorImager.

Quantitation of HBsAg, HBeAg, and human growth hormone was performed by radioimmunoassay (Abbott), enzyme immunoassay (Abbott), and radioimmunoassay (Nicholls), respectively. These assays were always performed within the linear range of the assay systems, as defined by dilutional studies.

To measure total cellular protein synthesis, transfected cells were transferred into medium with 25% of the usual amount of leucine and 20 μCi of [14C]leucine (DuPont NEN) at the end of the 6-h transfection period. At 48 h after the start of transfection, the cells were washed in saline and dissolved in denaturing protein sample buffer containing SDS and β-mercaptoethanol. An aliquot of the total cell extract was electrophoresed on a 10% polyacrylamide-SDS gel, which was dried and subjected to autoradiography and PhosphorImager scanning. To measure total cellular DNA synthesis, transfected cells were transferred into medium containing 20 μCi of [3H]thymidine at the end of the 6-h transfection period. At 48 h after the start of the transfection, the cells were washed in saline, and total DNA was extracted with standard protocols (Sambrook et al., 1989). An aliquot of the total DNA was added to scintillation fluid, and the amount of radioactivity was quantitated in a liquid scintillation counter.

The amount of released virions was assessed by the endogenous polymerase assay, modified from Bruss and Ganen (1991). Briefly, media from transfected cells were preclarified at 3,500 rpm (3,000 x g) for 30 min in a Sorvall HSM 4 rotor. The supernatants were recentrifuged at 50,000 rpm (106,000 x g) for 2 h in a Beckman TLA100.3 table-top ultracentrifuge rotor. The pellets were dissolved in a buffer containing 70 mM Tris-HCl, pH 7.5, 40 mM NH4Cl, 10 mM MgCl2, 0.5% Nonidet P-40, 1% β-mercaptoethanol, and 0.4 mM each of dATP, dGTP, and TTP. After vigorous vortexing, 10 μl of [α-32P]dCTP (3,000 Ci/mmol) was added, and the mixture was incubated at 37 °C overnight. Proteins were digested with proteinase K, and the elongated HBV genome DNA molecules were extracted with phenol-chloroform, precipitated with isopropanol, electrophoresed on a 3% agarose gel, and visualized by autoradiography.

RESULTS

Effect of Hydrogen Peroxide on HBV Virion Production—To determine whether hydrogen peroxide had an effect on the release of virion particles from host cells, we transiently transfected HuH-7 hepatoma cells with pHBV2, a plasmid containing the entire HBV genome that is capable of giving rise to all known viral transcripts and proteins and, hence, effects the release of virion particles from transfected cells. As the assay for virion production, we utilized the endogenous DNA-polymerase activity of HBV virions. HBV particles from transfected cells treated with various concentrations of hydrogen peroxide were collected by centrifugation, permeabilized with detergent, and incubated with deoxynucleotides, including [32P]dCTP. The resulting newly synthesized, full-length HBV DNA molecules were then electrophoresed and visualized by autoradiography. As shown (see Fig. 1A), upon hydrogen peroxide treatment, there was a dose-dependent decrease in the amount of the HBV DNA synthesized by the endogenous polymerase activity. Quantitation by a PhosphorImager revealed the decrease to be approximately 5-fold (see Fig. 1B). This decrease was due not to a direct effect of hydrogen peroxide on the viral polymerase, since adding hydrogen peroxide directly to the medium at the time of harvest had no effect on the assay results (data not shown). Glutathione largely prevented this decrease in HBV

\[ \text{Relative Amount of Polymerase Activity} = \frac{[\text{Activity in Control}]}{[\text{Activity in Treatment}]} \]
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FIG. 2. Map of a linearized representation of the HBV genome and the major transcripts (reviewed by Yen (1993)). HBeAg is a product of the full-length core open reading frame contained in the largest core (C) promoter transcript. The smaller core promoter transcripts are translated into the nucleocapsid (Core) protein, which initiates at the second ATG codon (*) in the core open reading frame and, less efficiently, into the viral polymerase. Similarly, the surface open reading frame gives rise to three co-linear forms of HBsAg, the majority of which comprises the middle and small forms. These forms are initiated from ATG codons (*) contained in mRNA transcribed from the surface (S) promoter. The upstream preS1 promoter is very weak; hence, the amount of full-length (large) HBsAg synthesized is very small.

FIG. 3. Effect of hydrogen peroxide on HBV gene expression. Huh-7 cells were co-transfected with pHBV2 (containing a dimer of HBV genome) and pXGH5 (containing the mouse metallothionein I promoter driving the human growth hormone gene) and then exposed to the indicated concentration of hydrogen peroxide. On the second day, the cells and media were harvested and assayed for HBsAg, HBeAg, and growth hormone. The results shown are mean ± S.D. of three independent transfections.
particle release (see Fig. 1A), while diamide, a depletor of intracellular glutathione, had the same effect as hydrogen peroxide (data not shown). Therefore, hydrogen peroxide must have exerted its effect by placing these cells under oxidative stress and not by any other chemical or physical properties.

Effect of Hydrogen Peroxide on HBV Gene Expression—Hydrogen peroxide may have decreased HBV virion production by decreasing the synthesis of one or more of its components or by blocking a stage in the morphogenesis or exocytosis of virion particles. To distinguish between these possibilities, we measured the effect of hydrogen peroxide on the amount of secreted HBsAg and HBeAg (these are products of the two major structural genes, the surface and core genes, respectively) (Fig. 2). As seen in Fig. 3, A and B, there was a dose-dependent decrease in the secretion of both antigens with hydrogen peroxide treatment.

We can rule out trivial mechanisms that might have explained this decrease in HBV protein secretion. First, it was possible that hydrogen peroxide may interfere with the assay systems. However, directly adding hydrogen peroxide to a sample with a known amount of either antigen had no measurable effect on the assay results (data not shown). Second, the effect cannot be attributed to a failure in secretion, since there was no corresponding accumulation of intracellular surface proteins with hydrogen peroxide treatment (Fig. 3D). Third, there might have been a general failure of transcription and/or translation because of toxic effects of hydrogen peroxide, but this cannot be the case, since the amount of [14C]leucine incorporated into total cellular proteins during the entire period of hydrogen peroxide treatment was only slightly lower than in control cells (Fig. 4A). In addition, the pattern of total host protein synthesized, as assessed by gel electrophoresis of [14C]leucine-labeled proteins, was grossly unaltered by hydrogen peroxide (Fig. 4B). Hydrogen peroxide did cause a small decrease in [3H]thymidine incorporation into cellular DNA (Fig. 4C), as expected from its ability to inhibit cell cycle progression (Shibanuma et al., 1991), but the difference (17%) was not sufficient to account for the difference in viral protein secretion. Furthermore, other groups have previously shown that hydrogen peroxide, at up to 300 μM concentration, has no significant effect on the transcription of such housekeeping genes as a-tubulin and glyceraldehyde phosphate dehydrogenase (Devary et al., 1991; Keyse and Emslie, 1992; Nose et al., 1991), and 500 μM hydrogen peroxide has been reported to be nontoxic to endothelial cells (Siflinger-Birnboim et al., 1992). Finally, this effect cannot be an artifact of transient transfections, since secretion of growth hormone directed by the mouse metallothionein I promoter (Fig. 3C) or by the thymidine kinase promoter (data not shown) in a co-transfected plasmid was not significantly affected. Furthermore, stably transfected HuH-7 cells responded in a similar manner (data not shown). Therefore, we conclude that hydrogen peroxide has a specific effect on the expression of HBV surface and e antigens.

It should be noted that pretreatment of culture medium with hydrogen peroxide for 1 h had no effect on HBV gene expression from transfected HuH-7 cells (data not shown); conversely, cells exposed to hydrogen peroxide still synthesized decreased amounts of HBV proteins, even when fresh medium was added 1 h after treatment (data not shown). Hence, hydrogen peroxide is not merely destroying a nutrient factor important for HBV gene expression nor generating a factor in the medium that is inimical to viral gene expression. Rather, it must be directly affecting a cellular component that directly or indirectly controls viral gene expression.

Hydrogen Peroxide Regulates Surface and Core Gene Transcription—To determine whether oxidant regulation of surface antigen synthesis was a reflection of changes in steady-state mRNA levels, we quantitated the amount of surface gene messages in transfected HuH-7 cells. As shown in Fig. 5A, primer extension analysis revealed a significant decrease (approximately 3-fold) in the levels of all the major transcripts upon hydrogen peroxide treatment. In contrast, no significant change in the amount of metallothionein promoter-driven growth hormone message, derived from the co-transfected plasmid pXGH-5, was found in the same cells (Fig. 5C). Similarly, the amount of cellular β-actin mRNA was similar in both sets of cells (data not shown). The presence of reduced glutathione at the time of hydrogen peroxide treatment prevented the de-
Hydrogen peroxide is a biologically relevant oxidant, which is released in large amounts by certain inflammatory cells (reviewed by Badwey and Karnovsky (1980)). Although it is difficult to relate the concentration of hydrogen peroxide used in our experiments to those present in areas of inflammation in vivo, it is likely that we are well within the pathologically relevant range, since we are using only a single pulse of hydrogen peroxide that is sub-lethal. In contrast, enough hydrogen peroxide can be generated by inflammatory cells to kill many different cell types (reviewed by Badwey and Karnovsky (1980)). In addition, it is possible that the HuH-7 hepatoma cells may react differently to oxidative stress from normal hepatocytes in situ. This is an unlikely scenario, since protection from oxidizing compounds is a basic requirement of all cells growing under aerobic conditions. Therefore, we believe that the results presented here are relevant to HBV infection in vivo. Nevertheless, it will be important to confirm our findings in intact animals, such as transgenic mice.

Gilles et al. (1992) have recently shown that in the intact liver, certain inflammatory cytokines, such as tumor necrosis factor α, can reduce the amount of all major HBV mRNA by up to 5-fold by increasing their degradation. Therefore, in areas of active hepatitis, HBV gene expression can be almost totally shut off by inflammatory mediators and products, via at least two different mechanisms. It seems highly unlikely that this dramatic effect is of no importance to the viral life cycle. However, at present we can only speculate on its pathophysiological significance. On the one hand, turning off viral gene expression may be one way for the body to rid itself of HBV infection. On the other hand, the virus may have evolved this mechanism to evade the host immune response. Since cytotoxic T-lymphocytes directed against HBV surface and core gene products are thought to be critical in the host defense mechanism (Mondelli et al., 1982; Penna et al., 1991, 1992), the shut-off of viral antigen expression in infected hepatocytes adjacent to an area of active inflammation may allow a few of those cells to escape

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With pHBV2 and pXGH5 and treated with water, 250 peroxide, and/or primer extension for surface gene transcripts and metallothionein promoters. Indicate the location of size markers. All products are of the expected reactions on crease in HBV surface gene mRNA levels (Fig. 5A). Therefore, a decrease in the amount of steady-state mRNA is at least partially responsible for the down-regulation of surface antigen synthesis by hydrogen peroxide.

Hydrogen peroxide also decreased the amount of all core gene transcripts by approximately 4-fold (Fig. 5B). It should be noted that the largest core transcript gives rise to the secreted HBeAg (see Fig. 1), while the smaller transcripts give rise to intracellular core protein, which is utilized as the viral nucleo-
antigen-specific killing. Since the HBV DNA would still be present in those cells, presumably viral expression and replication can proceed anew once the inflammation subsides. Indeed, there is evidence that HBV can evade the immune system and set up chronic infections in spite of a detectable cellular immune response against viral antigens (Penna et al., 1991). It may also be instructive to note that measles virus transcription in chronically infected neural cells is down-regulated by extracellular neutralizing antibodies and that this phenomenon has been postulated to be important for viral persistence in subcellular neutralizing antibodies and that this phenomenon has been postulated to be important for viral persistence in subacute sclerosing pan-encephalitis (Schneider-Schaulies et al., 1992). Therefore, we currently prefer the idea that down-regulation of HBV gene expression by oxidants is favorable for viral persistence, but further experiments in animal models will be needed for confirmation of this hypothesis.

In summary, we have described the oxidative down-regulation of HBV gene expression by a decrease in steady-state mRNA levels. Since reactive oxygen species have so far been described largely as activators of eucaryotic transcription, the compact HBV genome provides a suitable system for studying a novel aspect of gene regulation by oxidative stress that may lead to new insights on mammalian gene regulation in general.

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