HBV capsid (core) protein is a phosphoprotein that contains three major serine phosphoacceptor sites in its C-terminal domain. In our effort to investigate the potential site-specific and combinational roles of serine phosphorylation in HBV DNA replication, we found that the primary effect of core phosphorylation on HBV replication was on the pregenomic (pg) RNA encapsidation step. Further mechanistic studies revealed that the core phosphorylation state-dependent interaction between viral core and polymerase (pol) plays a critical role in HBV pgRNA encapsidation. It has been well documented that IFN-α prevents HBV pgRNA encapsidation in cell cultures, however, the underlying molecular mechanisms remain unclear. We report herein that IFN-α-elicited inhibition of HBV pgRNA encapsidation is associated with a loss of core/pol interaction without affecting the steady state level of either protein, indicating that IFN-α inhibits HBV pgRNA encapsidation through blocking core phosphorylation-dependent interaction with pol. Since cyclin-dependent kinase 2 (CDK2) was identified as a kinase for HBV core, we next analyzed the inductivity of CDK2 and its associated regulatory factors in IFN-α-treated cells. We found that a cellular CDK2 inhibitor, cyclin-dependent Kinase Inhibitor 3 (CDKN3), was significantly upregulated by IFN-α. We further demonstrated that overexpression of CDKN3 inhibited core/pol interaction and subsequent pgRNA encapsidation and DNA replication, which is reminiscent of IFN-α’s anti-HBV activity. What’s more, knockdown of CDKN3 in HBV replicating cells completely attenuated IFN-α-mediated inhibition of HBV core/pol interaction and pgRNA encapsidation. Taken together, CDKN3 is a host restriction factor for HBV replication through inhibition of viral nucleocapsid formation, and it plays a dominant role in IFN-α-elicited antiviral activity against HBV in cell cultures. The detailed profile of CDKN3-mediated alteration of HBV core phosphorylation in the context of IFN-α treatment is currently under investigation.