Lack of a Role of the Interferon-stimulated Response Element-like Region in Interferon α-induced Suppression of Hepatitis B Virus in Vitro*

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The antiviral effect of interferon-α (IFNa) on hepatitis B virus (HBV) is well documented in vitro and in vivo, but the mechanisms involved are elusive. Recently, an interferon-stimulated response like element (ISRE) competent for binding of interferon-stimulated gene factor-3 (p48) has been identified in the HBV enhancer I (1). Mutation of this element was shown to abrogate IFNa-mediated reduction of HBV X-gene promoter-driven reporter gene expression. This suggested a role of the ISRE and of p48 in IFNa-induced antiviral activity against productive HBV infection. Here, we analyzed the antiviral effect of both IFNa and enhanced p48 expression on complete HBV genomes containing the wild-type or mutated ISRE. In human hepatoma cells transfected with both genomes, viral RNA and replicative intermediates were reduced by IFNa treatment to a similar degree. Enhanced p48 expression increased IFNa-induced suppression of HBV RNA significantly from 75 ± 22.5% to 46 ± 9.8%, but this was independent of the integrity of the ISRE-like region. These data imply that p48 neither mediates the antiviral activity of IFNa against HBV nor down-regulates enhancer I activity by binding directly to the HBV ISRE-like region, but rather argue for an indirect role of p48.

Hepatitis B virus (HBV) is a small (3.2 kilobases) enveloped DNA virus, the replication of which involves reverse transcription of a pregenomic RNA in the cytoplasm within nucleocapsids, resulting in production of replicative DNA intermediates (1). From the cytoplasm, the nucleocapsids are either shuttled to the endoplasmic reticulum and converted to mature virions for transport out of the cell or to the nucleus for the establishment of a pool of covalently closed circular DNA (cccDNA) (2). The cccDNA in the nucleus serves as template for transcription of the pregenomic/C mRNA and subgenomic RNAs. The designation pregenomic/C mRNA derives from the fact that it serves both for translation of the core protein and as template for the generation of the viral DNA genome by reverse transcription.

Worldwide, more than 300 million people are chronically infected with HBV (3). Interferon-α (IFNa) treatment is one of the few effective therapies for chronic HBV carriers. However, it results in efficient reduction of the viral load only in 10–20% of treated patients and rarely, if at all, in complete virus elimination. So far, the mechanisms responsible for the IFNa-mediated reduction of the viremia in responding patients remain elusive. In vitro, IFNa-induced antiviral mechanisms against HBV have mostly been examined by using hepatoma cell lines expressing viral products from integrated or transiently transfected HBV DNA, since cell lines permissive for HBV infection are not available. Antiviral studies with IFNs in these systems led to intracellular inhibition of one or several HBV products depending on the type of cell line and system used (4–14). In the human hepatoma cell line Hep-HB107, which stably expresses HBV from a chromosomally integrated copy of the viral genome, IFNa treatment reduces the amount of intracellular replicative DNA intermediates, but affects neither viral polymerase activity within nucleocapsids nor the steady state levels of the viral RNAs (8). These data indicate that a posttranscriptional step of viral replication is targeted by IFNa-induced mechanisms, which might affect encapsidation of the pregenomic RNA or the stability of cytoplasmic nucleocapsids. On the other hand, IFNa-induced reduction of the viral RNA was observed in studies using a further stably transfected hepatoma cell line (HepG2.2.15) (5) and the transiently transfected human hepatoma cell line HuH7 (12). In HuH7 cells viral RNA levels were reduced posttranscriptionally by IFNa-induced mechanisms. The amount of replicative intermediates was more effectively reduced, suggesting that IFNa induces at least two independent antiviral activities acting on different levels (12). Furthermore, IFNa was reported to suppress the rate of transcription by down-regulating the activity of the HBV core promoter (13) and of the HBV enhancer I (14), as determined in reporter gene experiments with subgenomic HBV DNA fragments. Whether this also occurs in hepatoma cells producing complete HBV genomes has so far not been analyzed.

Taken together, it is well established that IFNa can induce an antiviral state against HBV in vitro, but neither the cellular proteins nor the IFNa-sensitive HBV targets involved are...
clear. In general, induction of an antiviral state in a cell by IFNα starts with the activation of the cytoplasmic protein tyrosine kinases Tyk2 and Jak1, and phosphorylation of latent cytoplasmic transcription factor subunits called STATs (signal transducer and activator of transcription) (15, 16). The STATs then assemble with interferon stimulated gene factor 3 (ISGF3), which translocates into the nucleus and binds to IFN-stimulated response elements (ISRE) located within the promoter region of IFNα- inducible genes.

Recently, in HuH7 cells treated with IFNα, a protein complex containing the transcription factor p48 was reported to interact with an ISRE-like region located within the enhancer I region of the HBV genome (11). Functional inactivation of the ISRE-like element that abrogated p48 binding in gel shift experiments abolished IFNα-induced suppression of HBV enhancer I activity in reporter gene expression experiments performed with subgenomic HBV DNA fragments. These results suggested that IFNα triggers direct binding of p48 to HBV DNA in concert with other proteins and thereby can modulate enhancer I activity. Since promoter and enhancer activities of HBV in part differ dramatically when full-length genomes or subgenomic fragments are tested (17), the relevance of these findings for IFNα-induced suppression of HBV propagation remains to be determined. This should be possible in hepatoma cells producing infectious virus when transfected with full-length HBV DNA. If IFNα treatment reduces the enhancer I activity also in this system and not only in reporter gene expression experiments, one should observe reduced levels of viral RNA and of other viral gene products (18). Since the HBV enhancer I is known to support synthesis of all viral transcripts in these cell lines (19), its modulation should affect all viral transcripts and subsequently all products synthesized from them.

In the present study, we investigated whether the ISRE-like region and p48 contribute to the antiviral activity induced by IFNα on HBV when propagated in human hepatoma cells. We demonstrate that inactivation of the ISRE-like region has no detectable effect on the IFNα-induced response in this system. Furthermore, overexpression of p48 enhanced IFNα-induced suppression of the viral RNA levels irrespectively of the mutation introduced. These data indicate that neither the ISRE-like region of the HBV nor direct binding of p48 to this element, if occurring at all in the full-length genome context, play a detectable role in the IFNα-induced antiviral state against HBV in hepatoma cells.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmid pHBV(wt) contains the HBV-DNA of subtype ayw (20) as head to tail linked dimer cloned via the EcoRI site (12). Plasmid pHBV ISRE(M) was produced by polymerase chain reaction-mediated oligonucleotide-directed mutagenesis. The mutation was introduced into pHBV-1 (plasmid containing the HBV-DNA as monomer, cloned via the EcoRI site) by amplification with Pco DNA polymerase (Roche Deutschland Holding GmbH, Grenzach-Wyhlen, Germany) and the complementary primer pairs HBV ISRE/M (5'-GCA GGC cC'T CAC CTT CTC GC-3'; only sense strand is shown, mutations are indicated by lowercase letters). Correct mutagenesis was verified by sequencing. The mutated HBV full-length fragment was isolated after EcoRI digestion by elution from an agarose gel. Thereafter, the fragment was inserted as head to tail dimer into the EcoRI site of pUC19. Plasmid pCMV/p48, which codes for human p48 under the control of a CMV promoter, was kindly provided by David E. Levy.

Cell Culture and Transfection Procedure—The human hepatoma cell line HuH7 was grown as monolayer in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Plasmid DNAs used for transfection were purified by ion exchange chromatography and were transfected using FuGENE 6 Transfection Reagent (Roche Deutsch- land Holding GmbH, Grenzach-Wyhlen, Germany) according to the protocol supplied. Cells (1.3 × 10⁶ cells/6-cm plate) were transfected with 2 μg of the corresponding HBV expression plasmids and 0.25 μg of pCMV/SEAP, which codes for a secreted alkaline phosphatase (SEAP). Plasmid pCMV/SEAP was cotransfected as control to monitor transfection efficiency and potential IFNα-mediated cytoxic effects. IFNα-2b (1,000 IU/ml, Intron A, Essex Pharma, Munich, Germany) was added 16 h after transfection, and cells were analyzed 48 h later. The amount of SEAP activity secreted into the medium of transfected cells was determined as described previously (12). For overexpression of p48 the pCMV/p48 expression plasmid was used, containing the p48 cDNA under control of a CMV promoter (21).

Purification of HBV-DNA from Intracellular Core Particles and of HBV RNA—Isolation of intracellular HBV-DNA was performed as described recently (22). Total RNA was prepared by use of the High Pure RNA Isolation Kit (Roche Deutschland Holding GmbH, Grenzach-Wyhlen, Germany) according to the protocol supplied.

Southern and Northern Blot Analysis—DNA isolated from cytoplasmic core particles were separated on an 1.2% agarose gel. DNAs and RNAs were blotted onto “Hybond N” nylon membranes (Nycomed Amersham, Buckinghamshire, United Kingdom) and hybridized with a 32P-labeled gel-purified full-length HBV DNA fragment. To standardize the loading, Northern blots were rehybridized with 32P-labeled glyceraldehyde-3-phosphate dehydrogenase-specific or actin-specific probes. HBV- and actin-specific probes were generated using a random-primed labeling kit (Nycomed Amersham, Buckinghamshire, United Kingdom). Glyceraldehyde-3-phosphate dehydrogenase-specific and actin-specific probes were generated by in vitro transcription using T7 RNA polymerase according to the manual supplied (Promega, Madison, WI). Blots were exposed to Fuji imaging screens, and signals were quantified by a Fujix BAS 2000 bio-imaging analyzer (Fuji, Tokyo, Japan) and by TINA software (Raytest, Straubenhardt, Germany).

Protein Extracts and EMSA—HuH7 cells were treated with 1,000 IU/ml IFNα for 3 h or left untreated. In addition, HuH7 cells were pretreated with 100 IU/ml IFNγ for 16 h and, thereafter, with 1,000 IU/ml IFNα for 30 min to increase the amount of activated ISGF3, as described previously (23, 24). Nuclear proteins were extracted as described previously (22). For EMSA, 15 μg of nuclear protein was preincubated for 5 min at room temperature with or without competitor in 25 μl of 20 mM HEPES (pH 7.9), 0.1% Nonidet P-40, 1 mM MgCl₂, 0.1 mM EDTA, 0.5 mM dithiothreitol, 8% glycerol, 2 μg of poly(dI-C), and 0.1 ng of an unrelated single-strand oligonucleotide (5'-ATGGTGGAGC-AAGGCGGAGGACG-3'). For the supershift experiments 2 μg of the indicated antibodies were added and incubated for 1 h at room temperature before addition of the labeled double stranded oligonucleotide probes. Polyclonal rabbit antiserum against STAT1 (p54/p91), STAT2, ISGF3-γ (p48), IRF1, and IRF2 were obtained from Santa Cruz Biotechnology, Inc. After addition of the indicated 32P-labeled double-stranded oligonucleotide probes and incubation for 30 min at room temperature, DNA-protein complexes were analyzed on 4% polyacrylamide gels (29:1) containing 25 mM Tris borate and 0.25 mM EDTA. For competition unlabeled double-stranded oligonucleotides were used in equimolar amounts, 10-fold, and 100-fold molar excess. The sequence of double-stranded oligonucleotides (only plus strand indicated) were used as probes and/or as competitors in EMSA were: HBV ISRE(wt), 5'-CGAGGCTTCACCTTCCGCG-3'; ISG15, 5'-GGGAAAGGGAAACCCGGAACTGAAGC-3'; C/EBPδ, 5'-CAAGAGAGAGTGCAG CT-3'; ATF-1, 5'-TACCTTGCAGCTCGGATGTGATG-3'.

RESULTS AND DISCUSSION

HBV enhancer I activity was shown to affect expression of all viral products (19), and its IFNα-mediated suppression was reported to depend on binding of p48 to an ISRE-like region (11). If p48 binding plays a major role in the antiviral activity of IFNα, as speculated previously, inactivation of the corresponding binding site should reduce IFNα-mediated suppression of HBV on the level of viral RNA and replicative intermediates in cells propagating HBV genomes (18). To test this we first wanted to confirm the ISRE-like region of the HBV ISRE-like region (Fig. 1A) inactivates p48 binding in an EMSA, as described recently for the corresponding oligonucleotides (11). We used nuclear extracts from IFNα-treated HuH7 cells or untreated cells and 32P-labeled double-stranded oligonucleotides of the HBV ISRE-like region (HBV-ISRE wt), the mutated HBV ISRE-like region (HBV-ISRE/M)), the ISRE region of the
cells with IFN lane 10 with an unrelated sequence (C) were used. from HuH7 cells pretreated with IFN like region. Nuclear extracts used for supershift analysis were prepared used for supershift experiments are indicated. nucelotide reduced formation of this complex (Fig. 1). Increasing amounts of the unlabeled HBV-ISRE(wt) oligo- 15 15 amounts of DNA-protein complexes (Fig. 1 B, lanes 2–6), or 100-fold molar excess of an oligonucleotide unlabeled double-stranded HBV ISRE(wt) (lanes 4–6), or HBV ISRE(M) (lanes 7–9), or an 100-fold molar excess of an oligonucleotide with an unrelated sequence (lane 10) were used. C, treatment of HuH7 cells with IFNγ and IFNα reveals binding of IFR 1 to the HBV ISRE-like region. Nuclear extracts used for supershift analysis were prepared from HuH7 cells pretreated with IFNγ for 16 h (100 IU/ml) and thereafter with IFNα for 30 min (1,000 IU/ml). Antisera (A) and probes used for supershift experiments are indicated.

FIG. 1. Binding of IFN-activated proteins to the HBV ISRE-like region is abrogated by mutations. A, scheme depicting the location of the ISRE-like region, viral open reading frames, and enhancer I within the HBV genome. Framed sequence regions indicate perfect match between the HBV ISRE(wt) and the consensus sequences of the ISRE (26) and of IRF-E (IRF element) (27) (N, any nucleotide; R, purine; S, G or C). Mutations introduced in HBV ISRE(M) to inactivate the previously reported binding of p48 are marked (bold/underlined). B, EMSA performed with nuclear extracts from HuH7 cells treated with 1,000 IU/ml IFNα for 48 h. To monitor transfection efficiency and potential IFNα-mediated cytotoxic effects cells were cotransfected with pCMV/SEAP. The amount of SEAP secreted into the medium (A) was determined by a SEAP enzyme activity assay. The levels of HBV transcripts (B) and of viral intracellular replicative DNA intermediates (C) were determined by Northern or Southern blotting, respectively. Quantitative evaluation of the data is shown in the adjacent panels. The SEAP activities are given as mOD/405 nm/min and the signal intensities of the blots as arbitrary units ± S.D. (n = 4).

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FIG. 2. Mutational inactivation of the ISRE-like region does not alter IFNα-mediated suppression of HBV transcription or replication. HuH7 cells transfected with pHBV(wt) or pHBV ISRE(M)-DNA were treated with or without 1,000 IU/ml IFNα for 48 h. As expected IFNα-mediated suppression of HBV transcription or replication. HuH7 cells transfected with pHBV(wt) or pHBV ISRE/M)-DNA were treated with or without 1,000 IU/ml IFNα for 48 h. To monitor transfection efficiency and potential IFNα-mediated cytotoxic effects cells were cotransfected with pCMV/SEAP. The amount of SEAP secreted into the medium (A) was determined by a SEAP enzyme activity assay. The levels of HBV transcripts (B) and of viral intracellular replicative DNA intermediates (C) were determined by Northern or Southern blotting, respectively. Quantitative evaluation of the data is shown in the adjacent panels. The SEAP activities are given as mOD/405 nm/min and the signal intensities of the blots as arbitrary units ± S.D. (n = 4).

IFN-stimulated gene 15 (ISG15), or the high affinity serum-inducible element sequence (Sie m67). As reported recently (11), IFNα treatment induced a pronounced increase in the amount of DNA-protein complexes (Fig. 1B, lanes 2, 3, 14, and 15). Increasing amounts of the unlabeled HBV-ISRE(wt) oligonucleotide reduced formation of this complex (Fig. 1B, lanes 4–6). In contrast, addition of the same amounts of the unlabeled HBV ISRE oligonucleotide with mutations (HBV-ISRE(M)) or of a control oligonucleotide unrelated to the ISRE had no effect (Fig. 1B, lanes 7–10). When using the mutated HBV ISRE-like element (HBV-ISRE(M)) as labeled probe for EMSA, no IFN-activated DNA-protein complexes were detected (Fig. 1B, lanes 16 and 17). As expected IFNα-activated DNA-protein complexes were detected using the ISRE of gene ISG15 (Fig. 1B, lanes 11 and 12) and the Sie m67 (Fig. 1B, lanes 18 and 19) oligonucleotide probes as positive controls. To further characterize the DNA-protein complexes bound to the HBV ISRE-like region, supershift experiments were performed with antisera against STAT 1, STAT 2, and p48. In these experiments a supershift of DNA-protein complexes was detected only with the Sie m67 oligonucleotide probe after addition of antiserum against STAT 1 (data not shown). Since neither binding of p48 to the HBV ISRE-like region nor binding of the ISGF3 complex (STAT 1, STAT 2, and p48) to the ISG15 ISRE were detected when using these extracts, we performed analogous experiments with nuclear extracts from HuH7 cells pretreated with IFNγ for 16 h before addition of IFNα. This treatment is known to increase the activation of ISGF3 (23, 24) and to induce binding of p48 to the HBV ISRE-like region (11). As expected, EMSA with these nuclear extracts showed supershifted signals with antibodies against STAT 1, STAT 2, p48, IRF1, and IRF2 when using the ISG15 (Fig. 1C, lanes 12–16) or the Sie m67 probes (Fig. 1C, lane 19), similar as published previously (23–25). When the mutated HBV-ISRE probe (HBV-ISRE/M) was used in these experiments, neither the same nor other DNA-protein complexes were observed (Fig. 1C, lanes 8 and 9). This rules out the possibility that binding of other...
transcription factors to the mutant oligonucleotide compensated for the IFN responsiveness of wt HBV ISRE. The IFN-induced protein-DNA complexes seen with the HBV-ISRE(wt) probe in our experiments did not contain p48 (Fig. 1C, lane 5) but did contain IRF 1 (Fig. 1C, lane 6). The latter result is consistent with a perfect IRF element (IRF-E) consensus sequence, known to bind IRF 1 and IRF 2, within the HBV ISRE-like region (Fig. 1A). Taken together, in contrast to a previous publication (11), in which binding of p48 to HBV ISRE wt sequence was claimed without including a specificity control for the anti-p48 antibodies used, our data indicate that p48 (although present and active in nuclear extracts from the same type of cells treated with IFNα or IFNγ plus IFNα) neither binds to the HBV-ISRE(wt) nor to the corresponding mutant oligonucleotide, while IRF 1 binds to the HBV-ISRE(wt), but not to the mutant oligonucleotide.

For functional analysis of the ISRE-like region in the complete HBV sequence context, we used a plasmid-integrated head to tail dimer of a fully functional HBV genome (pHBV wt) and in parallel the analogous construct containing mutations in the ISRE-like region (see above) of HBV (pHBV ISRE(M)), which abrogated binding of IFN-activated proteins in EMSA. These constructs were transfected into HuH-7 cells together with a plasmid pCMV/SEAP, which codes for a SEAP. Expression of SEAP was measured to control transfection efficiency and to exclude potential cytotoxic effects of IFNα.

Sixteen hours after transfection, cells were treated with 1,000 IU/ml IFNα or left untreated. Two days after addition of IFNα, the cell culture medium and the cells were harvested. Both DNA and RNA were extracted and analyzed by Southern and Northern blotting. In untreated cells, Northern blots revealed only a minor difference in the levels of the viral RNA produced by HBV wt and the mutant genome, indicating that the mutations had no significant effect on the enhancer activity (Fig. 2B). The slight increase of viral RNA in cells transfected with ISRE mutant virus DNA visible on the Northern blot reflects the corresponding higher transfection efficiency obtained with the mutant genome DNA compared with HBV wt DNA, as evident from the higher levels of SEAP (Fig. 2A). The amount of replicative DNA intermediates in pHBV ISRE(M)-transfected cells was about 5-fold lower than in cells producing wild-type HBV DNA, as determined by Southern blot analysis (Fig. 2C). This reduced replication efficacy is probably due to one of the three mutations introduced into the ISRE-like region causing an amino acid mutation (F656L) in the C-terminal end of the viral P-protein and the corresponding defect.

In cells treated with IFNα, the levels of viral RNA produced by HBV wt and mutant HBV were both decreased (Fig. 2B), indicating that inactivation of ISRE-like region did not abrogate the reduction of viral RNA levels by IFNα. Similarly, the amount of replicative DNA intermediates was also reduced by IFNα treatment both in the HBV wt and the HBV mutant.
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by Martin Forster.

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genome transfected cells (Fig. 2C). This was not due to a cyto-
toxic effect of IFNα, as SEAP expression levels were very sim-
ilar in treated and untreated cells (Fig. 2A). Independent of the functionality of the ISRE, the antiviral effect of IFNα was reproducibly weaker for viral RNA than for viral DNA (reduc-
tion to roughly 70% compared with 15%). This is consistent with our previously reported data, which suggested the exist-
ence of at least two antiviral mechanisms activated by IFNα, one acting on the RNA the other on the DNA level (12). Taken
together, these data argue against a significant role of the
ISRE-like element in IFNα-induced reduction of viral RNA and
replication in HBV producing hepatoma cells. One reason for the lack of a role of the ISRE-like region on replicating virus
may be that the ISRE-like region is covered by proteins, which
bind to adjacent regions not present in the reporter gene con-
structs used in previous studies (11, 14). Another reason may
be that in the full-length genome context efficient transcription of the HBV DNA from promoters located upstream of the ISRE-
like region prevents binding of IFN-activated proteins to the
ISRE-like region, while in the reporter gene constructs this
region is not transcribed. The major differences in promoter
and enhancer activities observed in many reported studies with
subgenomic HBV fragments compared with full-length HBV
DNA (17) is consistent with these interpretations.

Suppression of enhancer I activity by IFNα-induced p48
binding to the ISRE-like element in previous reporter gene
experiments was enhanced by overexpression of p48 in HuH7
cells (11). Therefore, we tested whether overexpression of p48
can increase IFNα-induced suppression of viral RNA and re-
replicative DNA intermediates in the full-length genome context.
HuH7 cells were cotransfected with pHBV(wt) and 0.5 or 2 µg
of pCMV/p48, treated with IFNα or left untreated, and ana-
alyzed as described above. As determined by Northern blotting,
IFNα treatment reduced the signal intensities for the viral
RNA to about 75% compared with untreated cells (Fig. 3).
Cotransfection of 0.5 or 2 µg of pCMV/p48 increased IFNα-
induced suppression of the viral RNAs to about 59 and 46%,
respectively (Fig. 3), as evident from quantitative evaluation of
the signals using a bioimager. The results indicate that en-
hanced expression of p48 increases the antiviral effect of IFNα
on the RNA levels in human hepatoma cells. On the level of
replicative DNA intermediates, an increase of IFNα-induced suppression was not observed with 0.5 µg of pCMV/p48 expres-
sion plasmid and only a modest, but significant, increase with
2 µg of pCMV/p48 compared with the antiviral activity induced by IFNα in the samples from cells not cotransfected with p48-
DNA (Fig. 4). Taken together, these data suggest that p48 is
present in limiting amounts in human hepatoma cells and
contributes to the IFNα-induced antiviral response against
HBV on the RNA and DNA level. To examine this further, and
to find out whether this contribution depends on the ISRE-like
region on HBV, we compared the IFNα-induced suppression on
the RNA level of HBV wt and ISRE mutant HBV under condi-
tions of enhanced p48 expression. HuH7 cells were cotrans-

tected with 2 µg of pCMV/p48 and pHBV wt or pHBV ISRE(M),
treated with IFNα or left untreated, and analyzed as described
above. Independent of the type of HBV genome transfected,
RNA levels were reduced to very similar levels by IFNα treat-
ment (Fig. 5). These data imply that even under nonlimiting
concentrations of p48, the binding of proteins activated by
IFNα to the ISRE-like region does not significantly contribute
to IFNα-induced reduction of the levels of HBV transcripts in
human hepatoma cells. However, our findings do not exclude a
function of the ISRE in infected hepatocytes in which the
template of transcription is cccDNA, which might allow binding
of IRF-1 and/or ISGF3 to the ISRE-like region. A suitable
animal system is required to investigate this further.
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