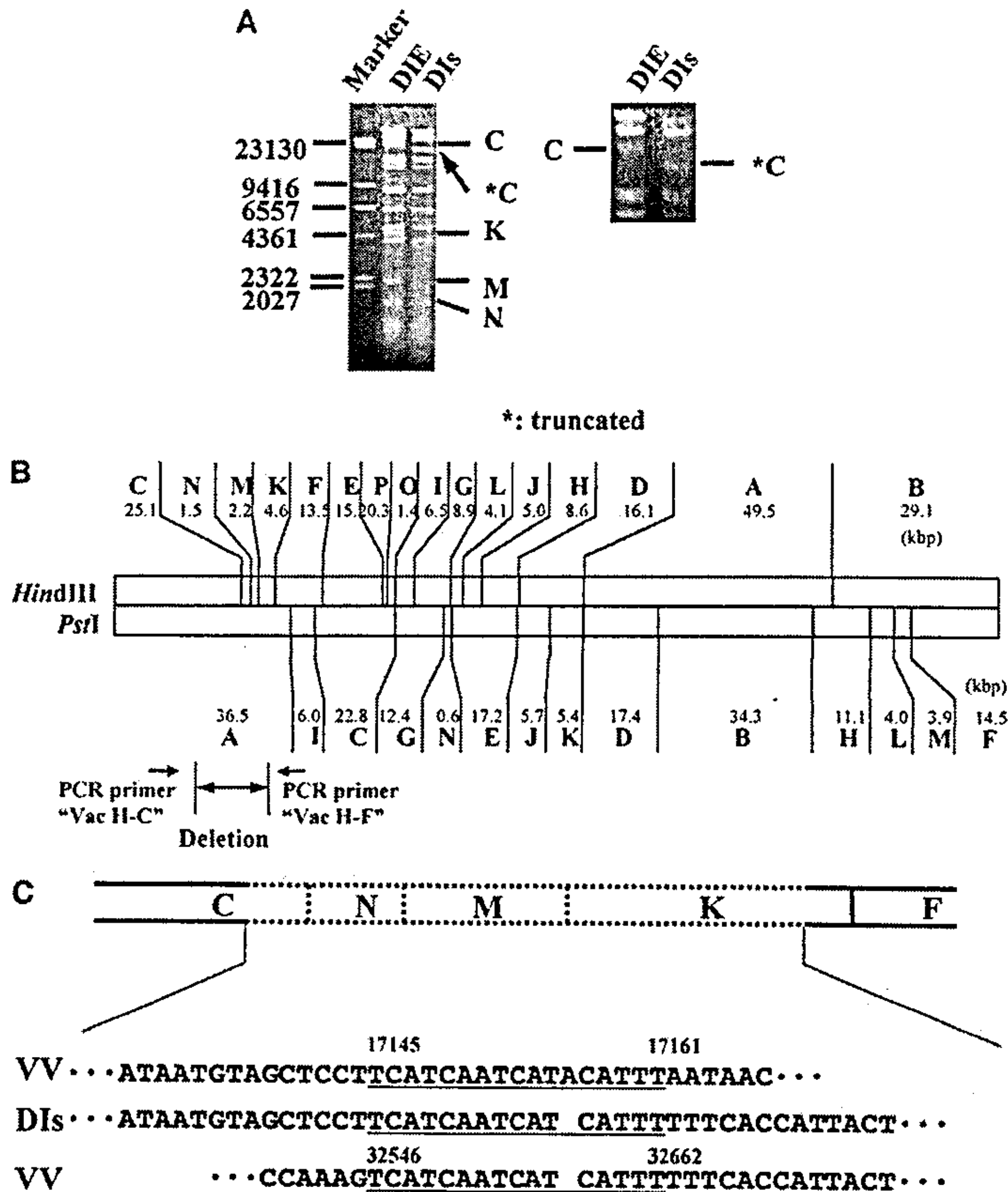
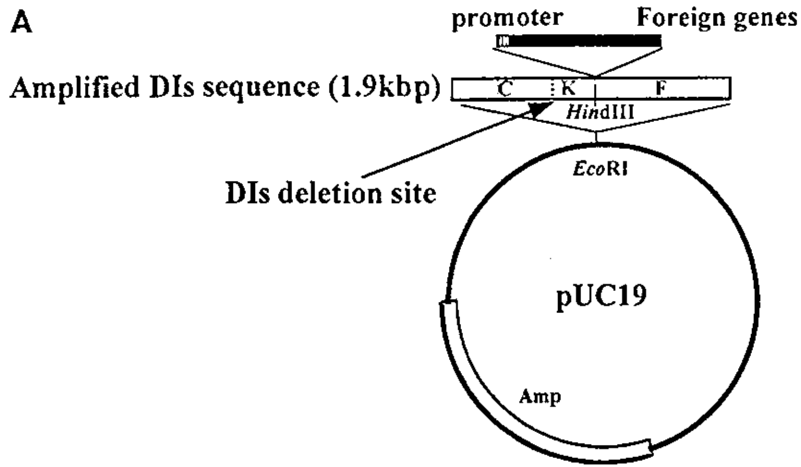
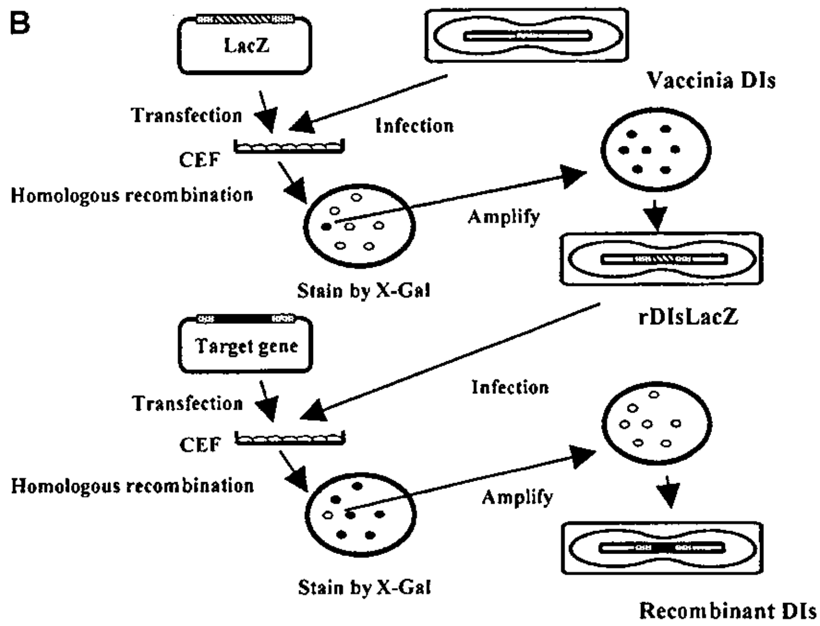


FIG. 1. Characterization of the genome of vaccinia virus DIs. (A) Electrophoretic patterns of DIs and DIE genomic DNA digested with *Hin*dIII. Fragments were run on 0.6% agarose gels (left) or 0.2% agarose gels (right) and labeled alphabetically in order of descending size. (B) Restriction map of the genome of the vaccinia virus Copenhagen strain. The locations of predicted cleavage sites of *Hin*dIII and *Pst*I are based on the DNA sequence of the entire genome (Goebel *et al.*, 1990). The fragments are numbered in order of decreasing size following the nomenclature of Mackett and Archard (1979) and DePhilippes (1982). The positions of PCR primers and the deleted region are indicated below. (C) Sequence analysis of the deleted region. The *Hin*dIII map and the junction sequences of vaccinia virus Copenhagen strain are shown. Numbers above the sequences represent the nucleotide positions from the 5′-terminus of the genome. The underlined region indicates the possible site of homologous recombination.

Stein *et al.*, 1989). To examine the translational properties of mRNA transcribed by T7 polymerase in mammalian cells, two plasmids (pT7Luc and pT7EMCLuc) were constructed. Each contained the T7 promoter, the luciferase gene, and the T7 terminator. pT7EMCLuc contained an additional IRES sequence of encephalomyocarditis virus (EMCV) under the T7 promoter (Aoki *et al.*, 1998). Previous results showed that transfection of pT7EMCLuc resulted in high levels of luciferase activity in various mammalian cell lines infected with either rAcCAT7 or rAdexCAT7, whereas only traces of luciferase expression could be detected when pT7Luc was transfected into cells (Aoki *et al.*, 1998; Yap *et al.*, 1997).

Interestingly, transfection of both pT7Luc and pT7-EMCLuc resulted in high levels of luciferase activity in all cell lines infected with rDIsT7pol (Fig. 3). The expression of pT7EMCLuc was higher than that of pT7Luc in all the cell lines tested. In contrast, the transfection of


A
promoter
Foreign genes
Amplified DIs sequence (1.9kbp)
C
K
F
HindIII
DIs deletion site
EcoRI
pUC19
Amp


B
LacZ
Vaccinia DIs
Transfection
Infection
CEF
Homologous recombination
Amplify
Stain by X-Gal
rDIsLacZ
Target gene
Transfection
Infection
CEF
Homologous recombination
Amplify
Stain by X-Gal
Recombinant DIs

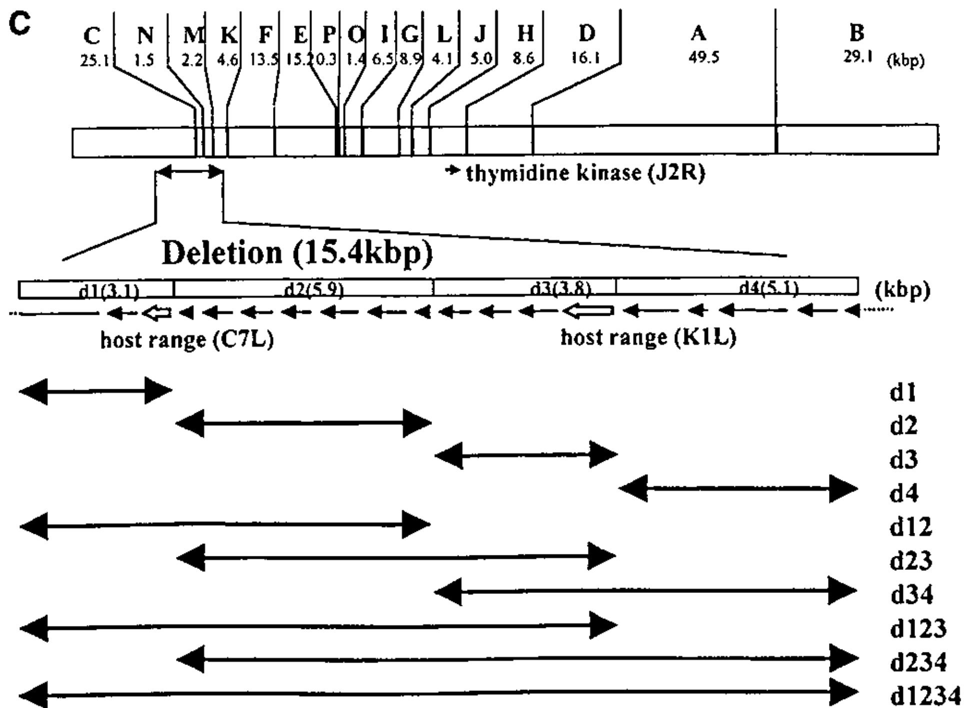

C
C 25.1
N 1.5
M 2.2
K 4.6
F 13.5
E 15.2
P 0.3
O 1.4
I 6.5
G 8.9
L 4.1
J 5.0
H 8.6
D 16.1
A 49.5
B 29.1
(kbp)
thymidine kinase (J2R)
Deletion (15.4kbp)
d1(3.1)
d2(5.9)
d3(3.8)
d4(5.1)
(kbp)
host range (C7L)
host range (K1L)
d1
d2
d3
d4
d12
d23
d34
d123
d234
d1234

TABLE 1

Replication Activity of Recombinant DIs Complemented with Fragments of the Deleted Region

	HeLa[a]	CV-1[a]	RK13[a]	CHO[a]	CEF[a]
DIE	7890	1578	842	<1	9474
d1234	5263	1405	589	N.D.	5263
d123	2485	337	130	N.D.	1479
d234	2349	557	805	N.D.	2161
d12	<1	<1	<1	N.D.	873
d23	1570	89	37	N.D.	364
d34	<1	<1	8	N.D.	527
d1	<1	<1	<1	N.D.	501
d2	1.1	<1	<1	N.D.	1305
d3	<1	<1	20	N.D.	406
d4	<1	<1	<1	N.D.	367
DIs	<1	<1	<1	N.D.	1600

[a] Cells were infected with DIE, DIs, or recombinant DIs at a m.o.i. of 0.05, incubated at 37°C, and harvested 48 h after infection. Viral input and yield titers were determined by plaque formation on CEF cells and the ratio of yield to input was calculated.

pT7EMCLuc after infection with rAdexCAT7 resulted in much higher levels of luciferase activity than the transfection of pT7Luc, as we have shown previously (Aoki *et al.*, 1998). rDIsT7pol and rAdexCAT7 produced no evident CPE at the indicated m.o.i. (data not shown). These results indicate that rDIsT7pol allows efficient expression of foreign genes under the control of the T7 promoter in the absence of a capping structure or IRES.

Recombinant DIs expressing full-length HIV-1 *gag*

The complete *gag* gene of HIV-1NL432 was then inserted into the *Hin*dIII site of pUC/DIs to generate pUC/DIsGag. Recombinant DIs virus expressing full-length HIV-1NL432 *gag* was then generated using rDIsLacZ with the transfer vector pUC/DIsGag and cloned as described above (Fig. 4A). Expression of HIV-1 Gag was detected by Western blot using cell lysates from CEF cells infected with rDIsBGag, while it was not detected in lysates from CEF cells infected with rDIsLacZ (Fig. 4B). On the other hand, rDIsBGag did not grow in mammalian cells such as HeLa, BHK, and RK13 (data not shown).

HIV-1 Gag-specific CTL was induced by intravenous inoculation of mice with 10^6 PFU of rDIsBGag. A control group of mice was treated with the same dose of rDIsLacZ. Gag-specific CTLs were induced in the immunized mice against BALB/c 3T3 cells pulsed with a mixture of HIV-1NL432 Gag peptides at effector-to-target ratios ranging from 50:1 to 12.5:1 (Fig. 5A). CTL activity was first detected at 2 weeks postinfection (p.i.) and was maintained at 12 weeks p.i. (Fig. 5C). In contrast, CTL activity was not detected in spleen cells from control mice inoculated with 10^6 PFU of rDIsLacZ (data not shown), nor was it detected against controls using allogeneic DAP.3 L cells as targets in cytotoxicity assays (Fig. 5B). In order to investigate direct CTL activity, we used spleen cells that were not stimulated with a mixture of HIV-1NL432 Gag peptides. The spleen cells showed a significant level of CTL activity specific for HIV-1 clade B Gag peptides, suggesting that rDIsBGag immunization directly induces CTL activity in animals (Fig. 6).

DISCUSSION

Forty years ago, the DIs strain, which replicates well only in CEF, was isolated from the DIE strain by successive 1-day egg passages and characterized by the production of tiny pocks on chicken CAM (Kitamura *et al.*, 1967; Tagaya *et al.*, 1961). In this study, we identified one large deletion by *Hin*dIII mapping and confirmed it by sequence analysis. However, we cannot rule out the possibility of additional minor deletions in other regions of the DIs genome, particularly in the right end. The *Hin*dIII fragments of this region (fragments A and B) are too large for molecular size comparison. To further characterize the deletions we thus performed *Pst*I digestion analysis and found that only one fragment of DIs migrated faster than that of DIE (Fig. 1A). Since the *Pst*I restriction map of the DIE strain is slightly different from that of the Copenhagen strain (Goebel *et al.*, 1990), it is difficult to determine the deleted regions based only on *Pst*I digestion analysis. However, we have already determined one large deletion in both *Hin*dIII mapping and sequence analysis. Therefore it is unlikely that there are any more major deletions in the right end of the DIs genome. Furthermore, we constructed recombinant DIs in which the entire 15.4-kbp deleted sequence was reintroduced. This recombinant virus could grow well not only in CEF, but also in other mammalian cells like HepG2, HeLa, 293T, and CV-1. The insertion of this deleted region was thus sufficient to restore the growth property of the DIs strain. The above results clearly suggest that the 15.4-kbp deletion of the left end of the DIs genome is responsible for the restriction of its host range. However, it should also be recognized that there

FIG. 2. Construction and generation of recombinant DIsT7pol. (A) Construction of the transfer vector pUC/DIs. The PCR-amplified DIs fragment was inserted into the *Eco*RI site of pUC19 as described. The dotted line between regions C and K indicates the location of the 15.4-kbp deletion in DIs. The *Hin*dIII site between regions K and F was utilized for the insertion of foreign genes. (B) Schematic representation of the protocol used to obtain recombinant DIs virus. (C) Complementation analyses of the deleted region. The region spanning the 15.4-kbp deletion in DIs was divided into four parts maintaining all ORFs (small arrows). Boxed arrows indicate the host range genes C7L and K1L. Ten fragments shown below were amplified by LA-PCR using vaccinia virus DIE as a template and inserted into the transfer vector pUC/DIs as described.

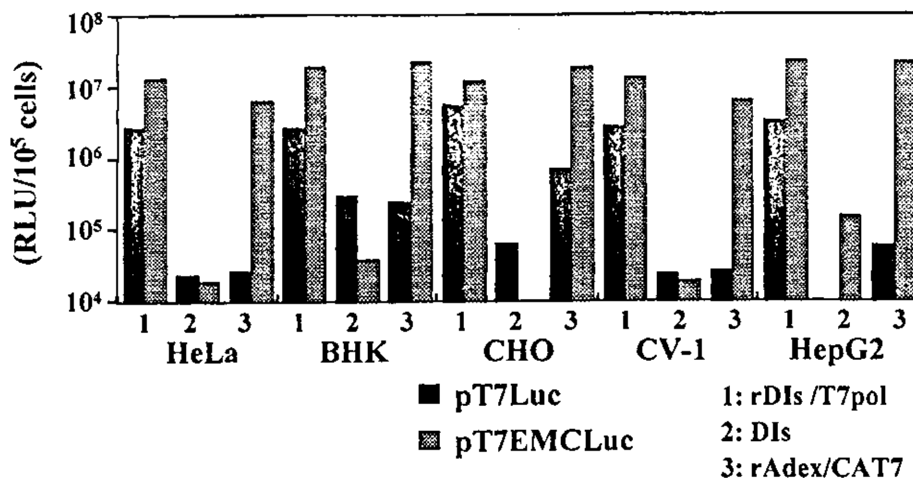


FIG. 3. Preparation of rDIsT7pol and expression of reporter plasmids in various mammalian cell line cells. HeLa, BHK, CHO, CV-1, and HepG2 cells were infected with rDIsT7pol (m.o.i. 2.0), DIs (m.o.i. 2.0), or rAdexCAT7 (m.o.i. 20.0) and transfected with luciferase reporter plasmid pT7Luc or pT7EMCLuc 24 h after infection. Luciferase activity was measured 24 h after transfection and expressed as relative light units (RLU) per 10^5 cells. The results shown are the mean of three independent experiments.

may be a number of undetected mutations that could well alter the functionality of some of the ORFs. For example, the failure to insert foreign genes within the TK locus raised the possibility of such mutations.

Genes of vaccinia virus determining its host range, including K1L (Gillard *et al.*, 1986; Perkus *et al.*, 1990), C7L (Oguiura *et al.*, 1993; Perkus *et al.*, 1990), and E3L (Beattie *et al.*, 1996; Chang *et al.*, 1995), have been identified and characterized. Restriction endonuclease and sequence analyses of the MVA genome have shown that a large deletion in the left end results in loss of the host range gene K1L (Antoine *et al.*, 1998). The functions of K1L and C7L are still unknown (Wyatt *et al.*, 1998), whereas E3L was shown to be the inhibitor of the interferon-induced 2′–5′A synthetase enzyme (Rivas *et al.*, 1998). Other ORFs (B5R, F13L, and A36R) are also known to be responsible for attenuation *in vivo*. These genes encode constituent proteins of the outer envelope of the virion and are critical for cell-to-cell and long-range spread of the virus (Blasco and Moss, 1991; Engelstad and Smith, 1993; Herrera *et al.*, 1998; Parkinson and Smith, 1994; Wolffe *et al.*, 1993).

Complementation analyses of DIs in this study suggest that the insertion of only the d3 region was insufficient to restore the growth character of parental DIE except growth in RK13 cells. This result is consistent with the previous observations (Carroll and Moss, 1997; Perkus *et al.*, 1990). When the d2 and d3 regions were complemented, the recombinant viruses grew efficiently in CV-1 and HeLa cells. Thus there is a gene(s) in the d2 region that supports extension of the host range gene K1L. Two proteins (C3L and N1L, termed virokines) secreted from infected cells with vaccinia virus were previously mapped in the d2 region (Kotwal and Moss, 1988) and are implicated in viral virulence (Kotwal *et al.*, 1989, 1990; Kotwal and Moss, 1988). Although further studies are necessary to identify the factor(s) that supports K1L function, it is possible that the absence of these genes results in further attenuation of K1L-defective vaccinia virus. In contrast, the C7L gene, located in the d1 region, did not act as a host range gene in the cells that we examined. It was previously shown that K1L and C7L behaved as equivalent genes in HeLa and MRC-5 cells (Oguiura *et al.*, 1993; Perkus *et al.*, 1990). In MVA, 37 ORFs including K1L are deleted or mutated but C7L is intact (Antoine *et al.*, 1998). However, restoration of the K1L gene in MVA failed to release the virus from its host restriction on mammalian cells (Carroll and Moss, 1997; Perkus *et al.*, 1990). Therefore, additional regulatory functions must be involved in allowing the progression of virus replication in mammalian cells. The ORFs in the d1 and d4 regions of DIs are not deleted in MVA, suggesting that these ORFs are not responsible for the host range restriction in DIs.

Vaccinia virus has been used extensively as a gene expression vector and furthermore as a recombinant vaccine. The major benefit of this system is its broad host range, which permits foreign gene expression in various mammalian cells and the possibility of inserting larger DNA fragments containing several genes at the same time. However, this system is not suitable for long-term analysis of the expressed products because of its extensive CPE derived from viral replication. In this context, highly attenuated vaccinia viruses (such as MVA and NYVAC) and DIs shown in this study are important, because they can express viral and inserted genes at high levels even in nonpermissive cells without showing CPE. It has been reported that foreign genes can be inserted into MVA by homologous recombination (Sutter and Moss, 1992), targeting precisely to the site of deletion to avoid any additional changes in phenotype.

As a first step in utilizing DIs as a nonreplicating viral

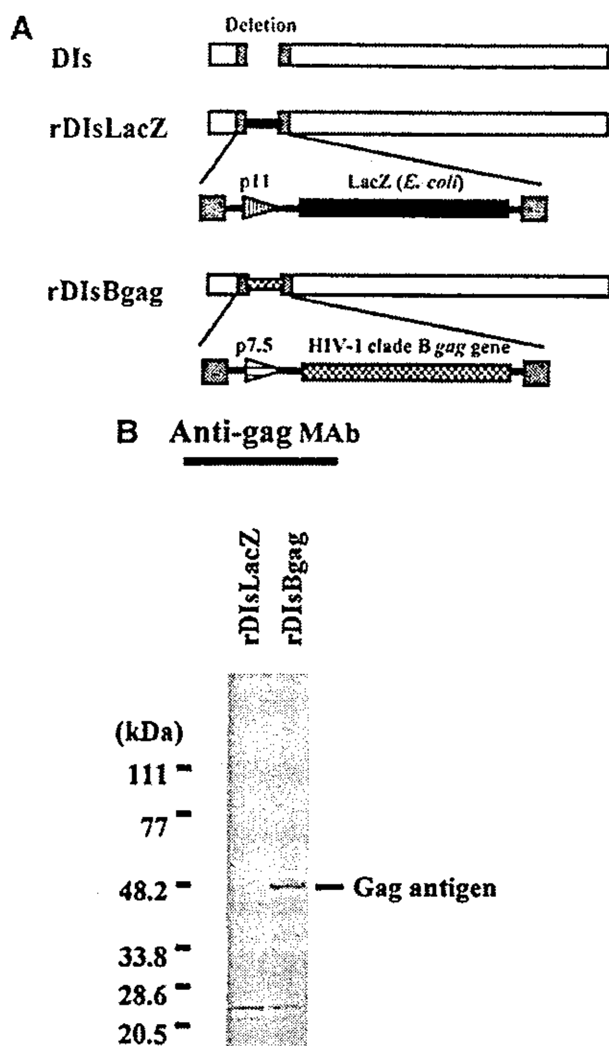


FIG. 4. Construction and expression of rDIs expressing the HIV-1 *gag* gene. (A) Schematic diagram of the construction of rDIsBGag and rDIsLacZ. (B) Detection of the HIV-1 Gag protein by Western blot using cell lysates from CEF cells infected with either rDIsBGag or rDIsLacZ as a control.

vector, we established a system to construct recombinant DIs. By a two-step selection procedure using the *E. coli lacZ* gene followed by X-Gal staining, we were able to easily select recombinant DIs that carried the desired target genes. We used this procedure to construct recombinant DIs expressing the bacteriophage T7 polymerase. The vaccinia virus/T7 polymerase transient expression system has previously proven to be useful for the synthesis of recombinant proteins in mammalian cells (Fuerst *et al.*, 1987; Moss *et al.*, 1990). In the vaccinia virus expression system, viral RNA polymerase facilitates the capping process of T7 transcripts in the cytoplasm, and transfected foreign genes under the T7 promoter can be efficiently translated (Ensinger *et al.*, 1975; Martin and Moss, 1975).

As shown in Fig. 3, when T7 polymerase was supplied by rDIsT7pol, the activity of a luciferase reporter gene translated from pT7luc was lower than that from pT7EMCLuc which carries EMCV IRES to allow cap-independent translation, as described previously (Elroy-Stein *et al.*, 1989). However, the expression level of the reporter gene was thought to be sufficient as a transient system because the luciferase activity of rDIsT7pol with

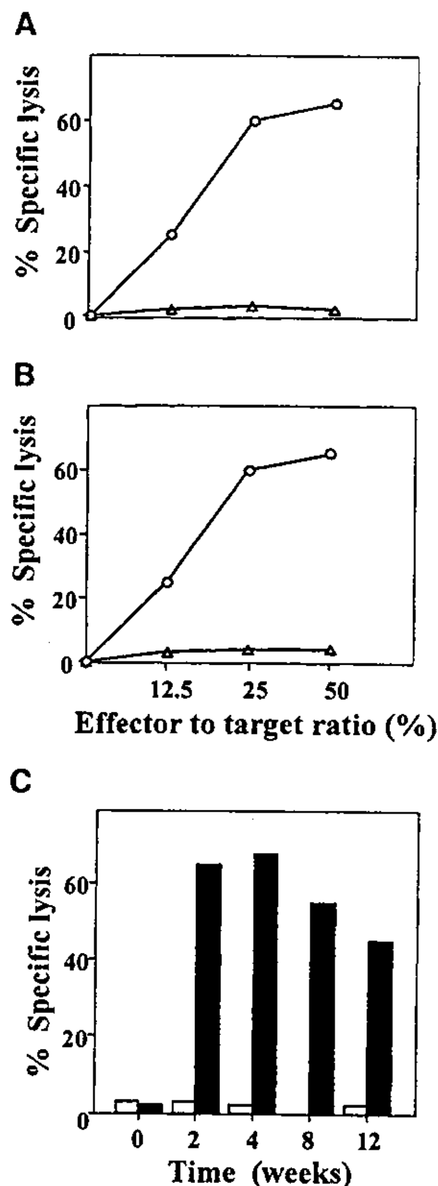


FIG. 5. Induction of HIV-1 Gag-specific CTL following inoculation of mice with rDIsBGag. BALB/c mice were intravenously inoculated with 10^6 PFU of rDIsBGag or rDIsLacZ as a control. Six weeks later, the animals were boosted with the same dose of antigen. (A) Cytolytic activity measured in spleen cells 2 weeks after the second inoculation and tested against BALB/c 3T3 cells with (O) or without (Δ) pulsing with a mixture of overlapping Gag peptides. (B) MHC class I restriction of CTL activity. Cytolytic activity was measured against BALB/c 3T3 cells (O) or DAP.3 L-cells (Δ) pulsed with Gag peptides. (C) Kinetics of induction of CTL activity measured 0, 2, 4, 8, and 12 weeks after the second inoculation with (black) or without (white) pulsing with a mixture of overlapping Gag peptides. The results shown are the mean of three independent experiments.

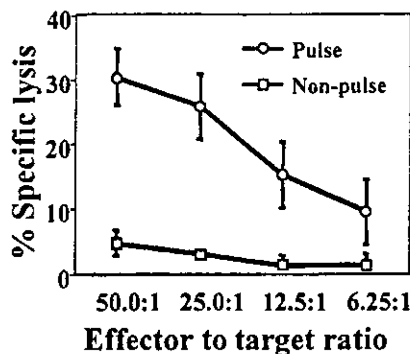


FIG. 6. Expression of direct CTL activity specific for HIV clade B Gag. Spleen cells from rDIsBGag-immunized mice were directly assayed for clade B Gag pooled peptide-specific CTL; pulse (○) and nonpulse (□). The results shown are the mean of three independent experiments. Vertical lines represent standard deviations.

pT7Luc was comparable to that of rAdexCAT7 with pT7EMCLuc. This result suggests that rDIsT7pol has the activity to add a capping structure on uncapped RNA and therefore undergoes translation as efficiently as RNA that contains EMCV IRES.

Elroy-Stein *et al.* (1989) previously showed that the T7 transcripts of vaccinia virus/T7 polymerase hybrid systems were mostly uncapped. This discrepancy might be due to the amount of transcribed RNA. In the earlier study, T7 transcripts accounted for as much as 30% of the total cytoplasmic RNA after infection with vTF7-3 (recombinant vaccinia virus carrying T7 polymerase, Fuerst *et al.*, 1987). Since rDIsT7pol has lost its capacity to grow in most of the mammalian cells tested, the amount of transcript was presumably much smaller than that generated by vTF7-3. Moreover, high levels of luciferase activity were detected in CHO cells in which wild vaccinia virus could not replicate, suggesting that rDIsT7pol could transiently supply T7 polymerase even to nonpermissive cells. It is known that transcription usually occurs in the nucleus after transfection of an expression plasmid. If the plasmid is transported to the nucleus, efficient transcription could be achieved under conditions where T7 RNA polymerase is functional in the nucleus. In addition, host cells also provide a nuclear capping enzyme by which T7 transcripts can be modified and lead to higher translation efficiency (Lieber *et al.*, 1989). Therefore, if we use rAcCAT7 or rAdexCAT7 for the transient expression system the target genes must be inserted under IRES so that cap-independent translation can be utilized. Otherwise we have to transport T7 polymerase to the nucleus and to add a capping structure on the 5′-terminus of transcribed RNA. The advantages of the DIsT7pol transient expression system are as follows: (i) only the transfection of target genes into the cytoplasm is sufficient for transcription by T7 polymerase because this enzyme is expressed in the cytoplasm; (ii) it is not necessary to install IRES upstream of the target genes because the capping structure can be added to the transcribed RNA by a viral capping enzyme; (iii) the cytopathic effect of DIsT7pol is dramatically reduced compared with that of other recombinant vaccinia viruses. In a different study, we used this DIsT7pol to examine the cell fusion activity of hepatitis C virus envelope proteins. T7 polymerase was eventually expressed and a long-term analysis of the expressed products was possible because no extensive cytopathic effects were induced (Takikawa *et al.*, 2000).

We show that the rDIs is completely replication-deficient in various mammalian cell lines, but is efficiently capable of expression of foreign genes, such as *E. coli lacZ* and bacteriophage T7 RNA polymerase. In spite of the deficiency in the replication of rDIs in all the mammalian cells used in this study, HIV Gag-specific CTL were obtained by intravenous inoculation of mice with rDIsBGag. Both $CD4^+$ cell loss and plasma viral copy number were significantly suppressed in immunized monkeys following challenge with pathogenic simian–human immunodeficiency virus SHIV-C2/1 (Sasaki *et al.*, 2000; Shinohara *et al.*, 1999; Y. Izumi *et al.*, unpublished observations). In earlier studies, MVA was used to express a variety of foreign genes, including β-galactosidase (Sutter and Moss, 1992), influenza virus hemagglutinin and nucleoprotein (Sutter *et al.*, 1994), bacteriophage T7 polymerase (Wyatt *et al.*, 1995), parainfluenza viruses HN and F (Wyatt *et al.*, 1996), HIV gp160 (Belyakov *et al.*, 1998), and SIV gag and pol (Seth *et al.*, 2000; Wyatt *et al.*, 1996). Some of these recombinant viruses were studied as candidate vaccines and appeared to be more effective than replication-competent vaccinia virus vaccines (Belyakov *et al.*, 1998; Nam *et al.*, 1999; Stittelaar *et al.*, 2000; Sutter and Moss, 1992; Sutter *et al.*, 1994). Further studies in macaques immunized with recombinant MVA expressing SIV Gag-Pol and/or Env showed comparable efficacy against challenge with pathogenic SIV (Amara *et al.*, 2001; Gotch *et al.*, 1991; Ourmanov *et al.*, 2000a,b; Seth *et al.*, 2000). rDIs exhibited no replicative ability and produced no infectious virions in these cells, indicating that the DIs strain may have a safety advantage when used as a recombinant vaccine vector. These results suggest that recombinant DIs-based constructs have potential as preventive vaccines against HIV-1.

In this study, we showed the usefulness of the DIs strain as a nonreplicating viral vector. This strain is also valuable for the analysis of the mechanism of host restriction of vaccinia virus. The limited replication of this strain in human cells is relevant to its potential use as a human vaccine. These observations will allow the design of effective, safe vaccinia-based recombinant vaccines.

MATERIALS AND METHODS

Cells and viruses

Human HeLa cells (ATCC CCL-2), HepG2 cells, 293T cells, rabbit kidney (RK13) cells (ATCC CCL-37), African green monkey (CV-1) cells (ATCC CCL-70), Chinese hamster ovary (CHO) cells, and baby hamster kidney (BHK) cells (ATCC CCL-10) were grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum (FCS). CEF cells were grown in Eagle's modified essential medium (EMEM) supplemented with 5% FCS. Vaccinia virus strain DIs was propagated in CEF cells. BALB/c 3T3 (H-2^d) and DAP.3 L cells (H-2^k) have been described previously (Takahashi *et al.,* 1988).

Animals

BALB/c (H-2^d) mice were obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan) and were used between 8 and 12 weeks of age.

Antibodies

Anti-T7 RNA polymerase rabbit serum was kindly provided by Dr. W. F. Studier (Brookhaven National Laboratory). The anti-HIV-1_{LAV} Gag monoclonal antibody V10 was kindly supplied by Dr. K. Ikuta (Research Institute for Microbial Diseases, Osaka University) (Matsuo *et al.,* 1992).

Plasmid constructions

Viral DNA from DIs was isolated as described (Wittek *et al.,* 1978), digested with *Hin*dIII or *Pst*I, separated on 0.6% agarose gels, and compared with the restriction map of the vaccinia virus Copenhagen strain (Goebel *et al.,* 1990). An approximately 1.9-kbp DNA fragment of the DIs genome, including the site of a 15.4-kbp deletion in the *Hin*dIII C, N, M, and K fragments (Fig. 1B), was amplified using a long and accurate polymerase chain reaction (LA-PCR) kit (Takara-Shuzo, Tokyo, Japan) and cloned into a pCR2.1 TA cloning vector (Invitrogen Japan, Tokyo, Japan). The sequence of the amplified region was determined using the primers Vac H-C (5′-ATAATGTAGCTCCTTCATCAATCATACATT) and Vac H-F (5′-AGGAGGTGGTGTAATAGACGAAGATTATAG).

An *Eco*RI fragment of pCR2.1 containing the amplified region of DIs was cloned into the same site of pUC19 and designated pUC/DIs. The amplified fragment contained one *Hin*dIII site corresponding to the site between K and F (Fig. 1B). This site was used as the cloning site for the insertion of foreign genes. The *E. coli lacZ* gene under the control of the vaccinia virus late promoter P11 was inserted into the *Hin*dIII site of pUC/DIs to generate the plasmid pUC/DIsLacZ. A 2.2-kbp DNA fragment containing the entire gene of bacteriophage T7 RNA polymerase (Aoki *et al.,* 1998) under the control of the vaccinia virus early/late promoter P7.5 was cloned into the *Hin*dIII site of pUC/DIs to generate the plasmid pUC/DIsT7pol. A DNA fragment encoding the full-length *gag* gene of HIV-1NL432 (nt 712–2498) under the control of the p7.5 promoter was inserted into the *Hin*dIII site of pUC/DIs to generate pUC/DIsGag. The positions of the nucleotides are numbered relative to the HIV-1HXB2 isolate (GenBank Accession No. K03455).

Complementation of the deleted region

To construct transfer vectors for the complementation of deleted regions, pUC/DIs was digested with *Hin*dIII, blunted, and ligated with a *Not*I linker to change the cloning site from *Hin*dIII to *Not*I, yielding pUC/DIsNot. The region corresponding to the deletion of DIs was divided into four parts and amplified, respectively, by LA-PCR using the DIE genome as a template. The primers used for PCR amplification were VacDIs del1-F (nt 16193–16222), (5′-AAGCGGCCGCCTACCAACCTACACTATTATATGATTATAG); VacDIs del2-F (nt 19384–19413), (5′-AAGCGGCCGCCTTAGTATAACACACATGTCTTAAAGTTTA); VacDIs del3-F (nt 25333–25362), (5′-AAGCGGCCGCGAGAATCAATACAAAAATTAATGCATCATT); VacDIs del4-F (nt 29183–29212), (5′-AAGCGGCCGCCCACCGTATCCATAAACAATATTAAGGAGA); VacDIs del1-R (nt 19346–19375), (5′-AAGCGGCCGCCTTATTCCATAGTAGCTTGTGGAATTTATA); VacDIs del2-R (nt 25287–25316), (5′-AAGCGGCCGCAAACTAAAATATGAATAAGTATTAAACATA); VacDIs del3-R (nt 29145–29174), (5′-AAGCGGCCGCATTATTTTATTTATATTGATGGGTACGTGA); and VacDIs del4-R (nt 34187–34216), (5′-AAGCGGCCGCAGGAGGTGGTGTAATAGACGAAGATTATAG). The positions of the oligonucleotides are numbered relative to the vaccinia virus Copenhagen strain (Goebel *et al.,* 1990) (GenBank Accession No. M35027).

Primer sequences were determined according to published sequences (Goebel *et al.,* 1990) and designed to avoid destruction of any ORFs. To insert the amplified fragments into pUC/DIsNot, an extra *Not*I site was added on the 5′-termini of the primers. Amplified fragments were cut with *Not*I and inserted into pUC/DIsNot. These transfer vectors were used for the construction of recombinant DIs using rDIsLacZ as the parental virus. The recombinant viruses were propagated in CEF cells. For analysis of virus replication, confluent monolayers of mammalian cells in 6-cm dishes were infected with recombinant virus at a m.o.i. of 0.05. After 1 h at 37°C, the cells were washed with PBS and incubated in fresh medium at 37°C. Cells were harvested at 0 and 48 h after infection, freeze-thawed, and sonicated, and virus replication was determined by dividing the virus titer at 48 h by that at 0 h.

Generation of recombinant DIs expressing bacteriophage T7 RNA polymerase

Infection was performed onto CEF cells grown in 8-cm dishes with DIs at a multiplicity of infection of 1.0. Trans-

fection was performed with 20 μg of pUC/DIsLacZ using Lipofectamine (GIBCO BRL/Life Technologies, Gaithersburg, MD). Recombinant DIs expressing β-galactosidase (rDIsLacZ) was selected by four consecutive rounds of plaque purification in CEF cells stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, Takara). rDIsLacZ was grown in CEF cells and used as the parental virus for recombination. Recombinant DIs expressing bacteriophage T7 RNA polymerase was generated using the homologous recombination method described above. CEF cells infected with rDIsLacZ were transfected with the transfer vector pUC/DIsT7pol. Recombinant virus that expressed T7 polymerase was selected by four consecutive rounds as colorless plaques when stained with X-Gal.

Transient expression of reporter genes

The activity of T7 polymerase was assayed using luciferase reporter constructs (pT7Luc and pT7EMCLuc) as described previously (Aoki *et al.*, 1998; Yap *et al.*, 1997). Monolayers of cells in 24-well plates were infected with DIs or rDIs-T7pol at an m.o.i. of 2.0. As a control, cells were infected with either recombinant baculovirus expressing T7 polymerase (rAcCAT7) (Yap *et al.*, 1997) or recombinant adenovirus expressing T7 polymerase (rAdexCAT7) (Aoki *et al.*, 1998) at a m.o.i. of 20. After 24 h, 1 μg of either pAcT7Luc or pAcT7EMCLuc was transfected using Lipofectamine. Luciferase activity was determined using the Pica Gene luciferase assay kit (Toyo Ink, Tokyo, Japan). Cells were harvested at 24 h posttransfection, washed twice with PBS, and lysed with 100 μl of the cell lysis buffer LUC/PGC-50 (Toyo Ink). For luciferase assays, 20 μl of cleared cell lysate was incubated with 100 μl of a reaction mixture containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzyme A, 470 μM luciferin, and 530 μM ATP. Relative light units (RLU) were measured using a luminometer (Berthold, Wildbad, Germany).

Production of recombinant DIs expressing full-length HIV-1 *gag*

Recombinant DIs virus expressing HIV-1_{NL432} *gag* was obtained using rDIsLacZ as the parental virus with the transfer vector pUC/DIsGag, and cloned as described above. The virus was grown in the CAM of 10-day eggs or in CEF cell cultures. Virus detection, culturing the recombinant clones, and Western blotting of the cell lysates were carried out as previously described (Morita *et al.*, 1977).

Generation of HIV-1 Gag-specific cytotoxic T-lymphocytes

The 20-mer overlapping peptides specific to the Gag sequence of HIV-1HXB2 were obtained from the AIDS Research and Reference Reagent Program (National Institutes of Health, Bethesda, MD). Twenty mice were intravenously inoculated with 10^6 PFU of either rDIsBGag or rDIsLacZ. After 8 weeks, the same dose of antigens was inoculated again. Two weeks later, spleen cells were harvested from the mice and stimulated with a 20 mM concentration of the peptide mixture for 5 days. The restimulated spleen cells were incubated with ^{51}Cr-labeled BALB/c 3T3 cells (H-2^d) or DAP.3 L cells (H-2^k) cells as targets for 4 h. The target cells were pretreated with ^{51}Cr for 60 min and then pulsed with a 20 mM peptide mixture for 60 min. The percentage of specific ^{51}Cr release was calculated as described (Takahashi *et al.*, 1988).

ACKNOWLEDGMENTS

We thank Drs. B. Moss, T. Suzuki, M. Minamitani, H. Takahashi, and Y. Nakagawa for helpful discussions. We also thank Ms. T. Mizoguchi for secretarial work and Ms. S. Ogawa for technical assistance. This work was supported in part by a grant-in-aid from the Organization for Drug ADR Relief, R & D Promotion, and Product Review to T.M. and the "Panel on AIDS" of the U.S.–Japan Cooperative Medical Science Program to M.H.

REFERENCES

Amara, R. R., Villinger, F., Altman, J. D., Lydy, S. L., O'neil, S. P., Staprans, S. I., Montefiori, D. C., Xu, Y., Herndon, J. G., Wyatt, L. S., Candido, M. A., Kozyr, N. L., Earl, P. L., Smith, J. M., Ma, H. L., Grimm, B. D., Hulsey, M. L., Miller, J., Mcclure, H. M., Mcnicholl, J. M., Moss, B., and Robinson, H. L. (2001). Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. *Science* **292**, 69–74.

Antoine, G., Scheiflinger, F., Dorner, F., and Falkner, F. G. (1998). The complete genomic sequence of the modified vaccinia Ankara strain: Comparison with other orthopoxviruses. *Virology* **244**, 365–396.

Aoki, Y., Aizaki, H., Shimoike, T., Tani, H., Ishii, K., Saito, I., Matsuura, Y., and Miyamura, T. (1998). A human liver cell line exhibits efficient translation of HCV RNAs produced by a recombinant adenovirus expressing T7 RNA polymerase. *Virology* **250**, 140–150.

Beattie, E., Kauffman, E. B., Martinez, H., Perkus, M. E., Jacobs, B. L., Paoletti, E., and Tartaglia, J. (1996). Host-range restriction of vaccinia virus E3L-specific deletion mutants. *Virus Genes* **12**, 89–94.

Belyakov, I. M., Wyatt, L. S., Ahlers, J. D., Earl, P., Pendleton, C. D., Kelsall, B. L., Strober, W., Moss, B., and Berzofsky, J. A. (1998). Induction of a mucosal cytotoxic T-lymphocyte response by intrarectal immunization with a replication-deficient recombinant vaccinia virus expressing human immunodeficiency virus 89.6 envelope protein. *J. Virol.* **72**, 8264–8272.

Blasco, R., and Moss, B. (1991). Extracellular vaccinia virus formation and cell-to-cell virus transmission are prevented by deletion of the gene encoding the 37,000-Dalton outer envelope protein. *J. Virol.* **65**, 5910–5920.

Brockmeier, S. L., Lager, K. M., Tartaglia, J., Riviere, M., Paoletti, E., and Mengeling, W. L. (1993). Vaccination of pigs against pseudorabies with highly attenuated vaccinia (NYVAC) recombinant viruses. *Vet. Microbiol.* **31**, 41–58.

Carroll, M. W., and Moss, B. (1997). Host range and cytopathogenicity of

the highly attenuated MVA strain of vaccinia virus: propagation and generation of recombinant viruses in a nonhuman mammalian cell line. *Virology* 238, 198–211.

Chang, H. W., Uribe, L. H., and Jacobs, B. L. (1995). Rescue of vaccinia virus lacking the E3L gene by mutants of E3L. *J. Virol.* 69, 6605–6608.

Elroy-Stein, O., Fuerst, T. R., and Moss, B. (1989). Cap-independent translation of mRNA conferred by encephalomyocarditis virus 5' sequence improves the performance of the vaccinia virus/bacteriophage T7 hybrid expression system. *Proc. Natl. Acad. Sci. USA* **86**, 6126–6130.

Engelstad, M., and Smith, G. L. (1993). The vaccinia virus 42-kDa envelope protein is required for the envelopment and egress of extracellular virus and for virus virulence. *Virology* **194**, 627–637.

Ensinger, M. J., Martin, S. A., Paoletti, E., and Moss, B. (1975). Modification of the 5'-terminus of mRNA by soluble guanylyl and methyl transferases from vaccinia virus. *Proc. Natl. Acad. Sci. USA* **83**, 2525–2529.

Fuerst, T. R., Earl, P. L., and Moss, B. (1987). Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes. *Mol. Cell. Biol.* **7**, 2538–2544.

Gillard, S., Spehner, D., Drillien, R., and Kirn, A. (1986). Localization and sequence of a vaccinia virus gene required for multiplication in human cells. *Proc. Natl. Acad. Sci. USA* **93**, 5573–5577.

Goebel, S. J., Johnson, G. P., Perkus, M. E., Davis, S. W., Winslow, J. P., and Paoletti, E. (1990). The complete DNA sequence of vaccinia virus. *Virology* **179**, 247–266, 517–563.

Gotch, F. M., Hovell, R., Delchambre, M., Silvera, P., and Mcmichael, A. J. (1991). Cytotoxic T-cell response to simian immunodeficiency virus by cynomolgus macaque monkeys immunized with recombinant vaccinia virus. *AIDS* **5**, 317–320.

Hel, Z., Venzon, D., Poudyal, M., Tsai, W. P., Giuliani, L., Woodward, R., Chougnet, C., Shearer, G., Altman, J. D., Watkins, D., Bischofberger, N., Abimiku, A., Markham, P., Tartaglia, J., and Franchini, G. (2000). Viremia control following antiretroviral treatment and therapeutic immunization during primary SIV251 infection of macaques. *Nature Med.* **6**, 1140–1146.

Herrera, E., Lorenzo, M. M., Blasco, R., and Isaacs, S. N. (1998). Functional analysis of vaccinia virus B5R protein: Essential role in virus envelopment is independent of a large portion of the extracellular domain. *J. Virol.* 72, 294–302.

Johnson, G. P., Goebel, S. J., and Paoletti, E. (1993). An update on the vaccinia virus genome. *Virology* **196**, 381–401.

Kazanji, M., Tartaglia, J., Franchini, G., De Thoisy, B., Talarmin, A., Contamin, H., Gessain, A., and De the, G. (2001). Immunogenicity and protective efficacy of recombinant human T-cell leukemia/lymphoma virus type 1 NYVAC and naked DNA vaccine candidates in squirrel monkeys (Saimiri sciureus). *J. Virol.* **75**, 5939–5948.

Kitamura, T., and Kitamura, T. (1963). Interference with the growth of vaccinia virus by an attenuated mutant virus. *Jpn. J. Med. Sci. Biol.* **16**, 343–357.

Kitamura, T., Kitamura, T., and Tagaya, I. (1967). Immunogenicity of an attenuated strain of vaccinia virus on rabbits and monkeys. *Nature* **215**, 1187–1188.

Konishi, E., Kurane, I., Mason, P. W., Shope, R. E., Kanesa-Thasan, N., Smucny, J. J., Hoke, C. H., Jr., and Ennis, F. A. (1998). Induction of Japanese encephalitis virus-specific cytotoxic T lymphocytes in humans by poxvirus-based JE vaccine candidates. *Vaccine* **16**, 842–849.

Konishi, E., Pincus, S., Paoletti, E., Laegreid, W. W., Shope, R. E., and Mason, P. W. (1992). A highly attenuated host range-restricted vaccinia virus strain, NYVAC, encoding the prM, E, and NS1 genes of Japanese encephalitis virus prevents JEV viremia in swine. *Virology* **190**, 454–458.

Kotwal, G. J., Hugin, A. W., and Moss, B. (1989). Mapping and insertional mutagenesis of a vaccinia virus gene encoding a 13,800-Da secreted protein. *Virology* **171**, 579–587.

Kotwal, G. J., Isaacs, S. N., Mckenzie, R., Frank, M. M., and Moss, B. (1990). Inhibition of the complement cascade by the major secretory protein of vaccinia virus. *Science* 250, 827–830.

Kotwal, G. J., and Moss, B. (1988). Vaccinia virus encodes a secretory polypeptide structurally related to complement control proteins. *Nature* 355, 176–178.

Lieber, A., Kiessling, U., and Strauss, M. (1989). High level gene expression in mammalian cells by a nuclear T7-phase RNA polymerase. *Nucleic Acids Res.* **17**, 8485–8493.

Mackett, M., Smith, G. L., and Moss, B. (1982). Vaccinia virus: a selectable eukaryotic cloning and expression vector. *Proc. Natl. Acad. Sci. USA* 79, 7415–7419.

Martin, S. A., and Moss, B. (1975). Modification of RNA by mRNA guanylyltransferase and mRNA (guanine-7-)methyltransferase from vaccinia virions. *J. Biol. Chem.* **250**, 9330–9335.

Matsuo, K., Nishino, Y., Kimura, T., Yamaguchi, R., Yamazaki, A., Mikami, T., and Ikuta, K. (1992). Highly conserved epitope domain in major core protein p24 is structurally similar among human, simian and feline immunodeficiency viruses. *J. Gen. Virol.* **73**, 2445–2450.

Mayr, A., Hochstein-Mintzel, V., and Stickl, H. (1975). Abstammung, Eigenschaften und Verwendung des attenuierten Vaccinia-Stammes MVA. *Infection* **3**, 6–14.

Morita, M., Aoyama, Y., Arita, M., Amona, H., Yoshizawa, H., Hashizume, S., Komatsu, T., and Tagaya, I. (1977). Comparative studies of several vaccinia virus strains by intrathalamic inoculation into cynomolgus monkeys. *Arch. Virol.* **53**, 197–208.

Moss, B. (2001). *Poxviridae. In* "Fields Virology" (B. N. Fields, D. M. Knipe, and P. M. Howley, Eds.), 4th ed., pp. 2849–2883. Lippincott–Raven, Philadelphia.

Moss, B., Elroy-Stein, O., Mizukami, T., Alexander, W. A., and Fuerst, T. R. (1990). Product review. New mammalian expression vectors. *Nature* **348**, 91–92.

Nam, J. H., Wyatt, L. S., Chae, S. L., Cho, H. W., Park, Y. K., and Moss, B. (1999). Protection against lethal Japanese encephalitis virus infection of mice by immunization with the highly attenuated MVA strain of vaccinia virus expressing JEV prM and E genes. *Vaccine* **17**(3), 261–268.

Oguiura, N., Spehner, D., and Drillien, R. (1993). Detection of a protein encoded by the vaccinia virus C7L open reading frame and study of its effect on virus multiplication in different cell lines. *J. Gen. Virol.* **74**, 1409–1413.

Ourmanov, I., Bilska, M., Hirsch, V. M., and Montefiori, D. C. (2000a). Recombinant modified vaccinia virus ankara expressing the surface gp120 of simian immunodeficiency virus (SIV) primes for a rapid neutralizing antibody response to SIV infection in macaques. *J. Virol.* **74**, 2960–2965.

Ourmanov, I., Brown, C. R., Moss, B., Carroll, M., Wyatt, L., Pletneva, L., Goldstein, S., Venzon, D., and Hirsch, V. M. (2000b). Comparative efficacy of recombinant modified vaccinia virus Ankara expressing simian immunodeficiency virus (SIV) Gag-Pol and/or Env in macaques challenged with pathogenic SIV. *J. Virol.* **74**, 2740–2751.

Parkinson, J. E., and Smith, G. L. (1994). Vaccinia virus gene A36R encodes a M(r) 43–50 K protein on the surface of extracellular enveloped virus. *Virology* **204**, 376–390.

Perkus, M. E., Goebel, S. J., Davis, S. W., Johnson, G. P., Limbach, K., Norton, E. K., and Paoletti, E. (1990). Vaccinia virus host range genes. *Virology* **179**, 276–286.

Rivas, C., Gil, J., Melkova, Z., Esteban, M., and Diaz-Guerra, M. (1998). Vaccinia virus E3L protein is an inhibitor of the interferon (i.f.n.)-induced 2-5A synthetase enzyme. *Virology* **243**, 406–414.

Sasaki, Y., Ami, Y., Nakasone, T., Shinohara, K., Takahashi, E., Ando, S., Someya, K., Suzaki, Y., and Honda, M. (2000). Induction of CD95 ligand expression on T lymphocytes and B lymphocytes and its contribution to apoptosis of CD95-up-regulated CD4+ T lymphocytes in macaques by infection with a pathogenic simian/human immunodeficiency virus. *Clin. Exp. Immunol.* **122**, 381–389.

Scheiflinger, F., Falkner, F. G., and Dorner, F. (1996). Evaluation of the

thymidine kinase (tk) locus as an insertion site in the highly attenuated vaccinia MVA strain. *Arch. Virol.* 141, 663–669.

Seth, A., Ourmanov, I., Kuroda, M. J., Schmitz, J. E., Carroll, M. W., Wyatt, L. S., Moss, B., Forman, M. A., Hirsch, V. M., and Letvin, N. L. (1998). Recombinant modified vaccinia virus Ankara-simian immunodeficiency virus gag pol elicits cytotoxic T lymphocytes in rhesus monkeys detected by a major histocompatibility complex class I/peptide tetramer. *Proc. Natl. Acad. Sci. USA* 95, 10112–10116.

Seth, A., Ourmanov, I., Schmitz, J. E., Kuroda, M. J., Lifton, M. A., Nickerson, C. E., Wyatt, L., Carroll, M., Moss, B., Venzon, D., Letvin, N. L., and Hirsch, V. M. (2000). Immunization with a modified vaccinia virus expressing simian immunodeficiency virus (SIV) Gag-Pol primes for an anamnestic Gag-specific cytotoxic T-lymphocyte response and is associated with reduction of viremia after SIV challenge. *J. Virol.* 74, 2502–2509.

Shinohara, K., Sakai, K., Ando, S., Ami, Y., Yoshino, N., Takahashi, E., Someya, K., Suzaki, Y., Nakasone, T., Sasaki, Y., Kaizu, M., Lu, Y., and Honda, M. (1999). A highly pathogenic simian/human immunodeficiency virus with genetic changes in cynomologus monkey. *J. Gen. Virol.* 80, 1231–1240.

Stittelaar, K. J., Kuiken, T., De Swart, R. L., Van Amerongen, G., Vos, H. W., Niesters, H. G., Van Schalkwijk, P., Van Der Kwast, T., Wyatt, L. S., Moss, B., and Osterhaus, A. D. (2001). Safety of modified vaccinia virus Ankara (MVA) in immune-suppressed macaques. *Vaccine* 19, 3700–3709.

Stittelaar, K. J., Wyatt, L. S., De Swart, R. L., Vos, H. W., Groen, J., Van Amerongen, G., Van Binnendijk, R. S., Rozenblatt, S., Moss, B., and Osterhaus, A. D. (2000). Protective immunity in macaques vaccinated with a modified vaccinia virus Ankara-based measles virus vaccine in the presence of passively acquired antibodies. *J. Virol.* 74, 4236–4243.

Sutter, G., and Moss, B. (1992). Nonreplicating vaccinia vector efficiently expresses recombinant genes. *Proc. Natl. Acad. Sci. USA* 89, 10847–10851.

Sutter, G., Wyatt, L. S., Foley, P. L., Bennink, J. R., and Moss, B. (1994). A recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus stimulates protective immunity in mice to influenza virus. *Vaccine* 12, 1032–1040.

Tagaya, I., Kitamura, T., and Sano, Y. (1961). A new mutant of dermovaccinia virus. *Nature* 192, 381–382.

Takahashi, H., Cohen, J., Hosmalin, A., Cease, K. B., Houghten, R., Cornette, J. L., Delisi, C., Moss, B., Germain, R. N., and Berzofsky, J. A. (1988). An immunodominant epitope of the human immunodeficiency virus envelope glycoprotein gp160 recognized by class I major histocompatibility complex molecule-restricted murine cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA* 85, 3105–3109.

Takikawa, S., Ishii, K., Aizaki, H., Suzuki, T., Asakura, H., Matsuura, Y., and Miyamura, T. (2000). Cell fusion activity of hepatitis C virus envelope proteins. *J. Virol.* 74, 5066–5074.

Tartaglia, J., Perkus, M. E., Taylor, J., Norton, E. K., Audonnet, J. C., Cox, W. I., Davis, S. W., Van Der Hoeven, J., Meignier, B., Riviere, M., Languet, B., and Paoletti, E. (1992). NYVAC: A highly attenuated strain of vaccinia virus. *Virology* 188, 217–232.

Wittek, R., Menna, A., Muller, H. K., Schumperli, D., Boseley, P. G., and Wyler, R. (1978). Inverted terminal repeats in rabbit poxvirus and vaccinia virus DNA. *J. Virol.* 28, 171–181.

Wolffe, E. J., Isaacs, S. N., and Moss, B. (1993). Deletion of the vaccinia virus B5R gene encoding a 42-kilodalton membrane glycoprotein inhibits extracellular virus envelope formation and dissemination. *J. Virol.* 67, 4732–4741.

Wyatt, L. S., Carroll, M. W., Czerny, C. P., Merchlinsky, M., Sisler, J. R., and Moss, B. (1998). Marker rescue of the host range restriction defects of modified vaccinia virus Ankara. *Virology* 251, 334–342.

Wyatt, L. S., Moss, B., and Rozenblatt, S. (1995). Replication-deficient vaccinia virus encoding bacteriophage T7 RNA polymerase for transient gene expression in mammalian cells. *Virology* 210, 202–205.

Wyatt, L. S., Shors, S. T., Murphy, B. R., and Moss, B. (1996). Development of a replication-deficient recombinant vaccinia virus vaccine effective against parainfluenza virus 3 infection in an animal model. *Vaccine* 14, 1451–1458.

Yap, C. C., Ishii, K., Aoki, Y., Aizaki, H., Tani, H., Shimizu, H., Ueno, Y., Miyamura, T., and Matsuura, Y. (1997). A hybrid baculovirus-T7 RNA polymerase system for recovery of an infectious virus from cDNA. *Virology* 231, 192–200.

Virology 304, 415–424 (2002)
doi:10.1006/viro.2002.1702

Alteration of Intrahepatic Cytokine Expression and AP-1 Activation in Transgenic Mice Expressing Hepatitis C Virus Core Protein

Takeya Tsutsumi,*,† Tetsuro Suzuki,* Kyoji Moriya,† Hiroshi Yotsuyanagi,† Yoshizumi Shintani,† Hajime Fujie,† Yoshiharu Matsuura,†,‡ Satoshi Kimura,† Kazuhiko Koike,† and Tatsuo Miyamura*,[1]

*Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan; †Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo, Japan; and ‡Research Center for Emerging Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

Received May 31, 2002; returned to author for revision June 24, 2002; accepted August 5, 2002

Hepatitis C virus (HCV) infection often leads to the development of hepatocellular carcinoma (HCC), but its molecular mechanism has not been clearly elucidated. Previously, transgenic mice constitutively expressing HCV core protein have been shown to develop HCC, suggesting a pivotal role of the core protein in hepatocarcinogenesis. Here, we analyzed the expression of cytokines associated with a variety of cellular processes, including cell proliferation, in the mouse model for HCV-associated HCC to define the molecular events prior to oncogenesis. The expression of tumor necrosis factor-α and interleukin-1β was increased at both protein and mRNA levels. In addition, the activities of c-Jun N-terminal kinase and activator protein-1 (AP-1), downstream effectors, were enhanced, while IκB kinase or nuclear factor-κB activities were not enhanced. Thus, the altered *in vivo* expression of cytokines with AP-1 activation in consequence to the core protein expression may contribute to hepatocarcinogenesis in persistent HCV infection. © 2002 Elsevier Science (USA)

Key Words: HCV; core protein; transgenic mouse; TNF-α; IL-1β; AP-1; JNK; NF-κB; HCC.

Hepatitis C virus (HCV) is a causative agent of acute and chronic non-A non-B hepatitis (Choo *et al.*, 1989). HCV infection frequently persists and leads to liver cirrhosis and hepatocellular carcinoma (HCC) (Saito *et al.*, 1990). However, the mechanism of hepatocarcinogenesis in HCV infection is not clearly understood.

The core protein of HCV, by interacting with its viral RNA, forms a viral nucleocapsid (Shimoike *et al.*, 1999). In addition to this genome-packaging function, the core protein has various functions. The core protein transforms fibroblasts with or without the cooperation of the *ras* oncogene, suppresses or enhances apoptosis of cultured cells, modulates the transcription of some genes, and binds to some cellular proteins (Lai and Ware, 2000; McLauchlan, 2000; Suzuki *et al.*, 1999). In addition, we have reported that transgenic mice expressing the core protein in the liver develop HCC (Moriya *et al.*, 1998). These results indicate that the core protein is closely associated with hepatocarcinogenesis in HCV infection.

Cytokines are important mediators of tissue injury and inflammation. The cytokine signal is transmitted to cells via membrane-bound receptors. This cytokine–receptor interaction activates intracellular signaling pathways. Cytokine-inducible transcription factors, including activator protein-1 (AP-1) and nuclear factor-κB (NF-κB), interact with their responsive elements in the promoter regions of various genes. Cytokines like tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6 are closely associated with liver regeneration. Treatment with antibodies to TNF-α before partial hepatectomy resulted in decreased DNA synthesis and an abrogation of increases in c-Jun N-terminal kinase (JNK), c-jun mRNA, and nuclear AP-1 activity (Diehl *et al.*, 1994). In addition, mice lacking the type I TNF receptor were deficient in liver regeneration (Yamada *et al.*, 1997). Concerning IL-6, hepatocyte DNA synthesis during liver regeneration was suppressed in mice carrying a homozygous deletion of the IL-6 gene (Cressman *et al.*, 1996). Taken together, the cytokines described above are considered as playing important roles in cell proliferation and, possibly, hepatocarcinogenesis.

TNF-α, IL-1, and IL-6 are mainly synthesized by blood cells like lymphocytes and monocytes, but the liver is also an important producer of these cytokines. Human and murine hepatocytes as well as Kupffer cells produce TNF-α, IL-1, and IL-6 (Andus *et al.*, 1991; Gonzalez-Amaro *et al.*, 1994; Hunt *et al.*, 1992; Wordemann *et al.*, 1998).

Since TNF-α signaling plays important roles in a wide range of cellular functions, including inflammation, cell proliferation, differentiation, and apoptosis, it is rational to consider that the core protein contributes to these

[1] To whom correspondence and reprint requests should be addressed at the Department of Virology II, National Institute of Infectious Diseases, 1-23-1, Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Fax: 81-3-5285-1161. E-mail: tmiyam@nih.go.jp.

0042-6822/02 $35.00
© 2002 Elsevier Science (USA)
All rights reserved.

pathogeneses by influencing TNF-α signaling pathways. In fact, several previous reports showed an association or interaction of the core protein with TNF-α signaling pathways (Chen *et al.*, 1997; Marusawa *et al.*, 1999; Matsumoto *et al.*, 1997; Shrivastava *et al.*, 1998; Zhu *et al.*, 1998). However, carcinoma or transformed cell lines were used to express the core protein, and some conflicting evidence was observed in these studies.

Here we demonstrate the enhancement of TNF-α and IL-1β expression in the liver of core-transgenic mice in the absence of inflammation in the liver. In these mice, the activation of JNK and transcription factor AP-1, which are downstream effectors of these cytokines, was also demonstrated. These results suggest that the core protein potentially enhances the hepatic expression of TNF-α and IL-1β and activates AP-1 *in vivo*, contributing to hepatocarcinogenesis.

RESULTS

HCV core protein modulates intrahepatic cytokine expression in the liver of core-transgenic mice

Cytokines such as TNF-α, IL-1β, and IL-6 are closely associated not only with controlling proliferation of hepatocytes, but also with various pathogeneses of the liver, including regeneration, necrosis, inflammation, fibrosis, and, possibly, carcinogenesis (Andus *et al.*, 1991). The liver is an important source of production of these cytokines as well as the main scavenger for them (Andus *et al.*, 1991). Therefore, we first investigated the gene expression profiles of the above cytokines in the liver of core-transgenic mice. In this study, we used pairs of young, 2- to 3-month-old transgenic mice and their littermates for the following reasons. First, we aimed to assess the direct *in vivo* effect of the core protein in the liver. Second, we wanted to use core-transgenic mice with histopathological changes of the liver that were as slight as possible without advanced steatosis or HCC because these pathological changes themselves might modulate cytokine expression. The levels of the core protein in the liver of core-transgenic mice were within the range of those in livers of HCV-associated HCC patients (Koike *et al.*, 2002).

We determined intrahepatic mRNA expression of cytokines by RT–PCR. Figure 1A shows the mRNA expression of TNF-α and IL-1β in the liver of core-transgenic mice of 2 to 3 months old and that of nontransgenic control mice of the same ages. The expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was also examined. In spite of a lack of inflammation in the liver of core-transgenic mice, gene expression of these cytokines was enhanced in core-transgenic mice compared to that of nontransgenic littermates. The expression levels of the GAPDH gene were similar in all of the samples, indicating that the amount of mRNA used and the efficiency of the amplification were almost equal. A similar tendency was also observed when we decreased the cycles of the PCR by five, indicating that the PCR products were not saturated. On the other hand, the gene expression of IL-6 as well as of β-actin in the liver of core-transgenic mice was principally similar to that of nontransgenic mice (Fig. 1B). Such modulation of the cytokine gene expression was observed in the mice from two independent lines. We further performed TaqMan real-time PCR to quantify TNF-α and IL-1β mRNA in the liver of mice. For statistical analysis, we examined three more pairs of mice in addition to those used for the above experiment. One hundred nanograms of total RNA was amplified, and the amount of the PCR products was recorded at each cycle. mRNA copy numbers calculated from the calibration curves were normalized against GAPDH, the expression of which was not altered by the core protein in mice (data not shown). As shown in Table 1, the mRNA levels of TNF-α and IL-1β in the liver of core-transgenic mice were increased 2- to 2.5-fold and 1.5- to 2-fold, respectively. These results suggest that the expression of the core protein enhances intrahepatic gene expression of the cytokines TNF-α and IL-1β in core-transgenic mice.

To assess whether these enhanced gene expressions reflected the intrahepatic protein levels, we next determined the protein expression of TNF-α and IL-1β in the liver of these mice. The amount of TNF-α and IL-1β was determined by ELISA using mouse liver lysates and divided by the amount of liver total proteins. As shown in Table 2, both the TNF-α and IL-1β protein levels were

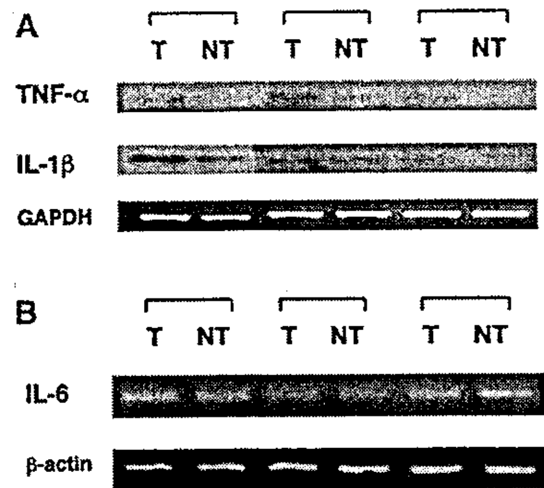


FIG. 1. mRNA expression of cytokines in the liver of core-transgenic (T) and nontransgenic (NT) mice by RT–PCR. Total RNA was extracted from the liver of mice and RT–PCR (30 cycles) was performed using adequate primers. (A) mRNA expression of TNF-α and IL-1β in three pairs of 2- to 3-month-old mice. The GAPDH gene was used as an internal control. After PCR products were separated on the gel, Southern blot analysis was performed to confirm an adequate amplification of TNF-α and IL-1β genes. (B) mRNA expression of IL-6 in the same pairs of as mice shown in (A). The β-actin gene was used as an internal control.

TABLE 1

mRNA Levels of TNF-α and IL-1β in the Livers of Transgenic and Nontransgenic Mice

	Transgenic mice (n = 6)	Nontransgenic mice (n = 6)	P[a]
TNF-α	1.30 (±0.74) × 10^{-2}[b]	0.54 (±0.13) × 10^{-2}	<0.05
IL-1β	2.25 (±0.72) × 10^{-2}	1.24 (±0.24) × 10^{-2}	<0.05

[a] Mann–Whitney's U test.
[b] Values are expressed in mRNA copy numbers quantified by real-time PCR, followed by normalization against levels of GAPDH (means ± SE).

TABLE 2

Protein Levels of TNF-α and IL-1β in the Livers of Transgenic and Nontransgenic Mice

	Transgenic mice (n = 7)	Nontransgenic mice (n = 7)	P[a]
TNF-α	600.5 (±311.0)[b]	323.4 (±114.6)	<0.05
IL-1β	1387.3 (±565.8)	610.6 (±160.6)	<0.05

[a] Mann-Whitney's U test.
[b] Picograms/milligrams total proteins (means ± SE).

significantly increased in the livers of core-transgenic mice compared to nontransgenic littermates. The ratios (core-transgenic/nontransgenic) of the TNF-α and IL-1β protein levels were 1.9 and 2.3, respectively, which were slightly different from those of the mRNA levels (2.4 and 1.8, respectively). We cannot clearly show the reason for this, but one possible explanation is that possible post-transcriptional modifications work for the protein expression of the cytokines as reported previously (Chantry *et al.*, 1989; Moller *et al.*, 1998). We also determined the protein expression levels of these cytokines in the livers of the mice expressing HCV envelope proteins under the same regulatory region as that of the core-gene-transgenic mice (Koike *et al.*, 1997) and found similar expression levels in envelope-transgenic and control mice (data not shown).

HCV core protein activates the JNK–AP-1 pathway in the liver of core-transgenic mice

TNF-α and IL-1β bind to their specific receptors on the cell surface and activate intracellular signaling cascades. One of the well-known pathways is JNK–AP-1; JNK is activated by TNF-α- or IL-1β stimulation and phosphorylates c-Jun, leading to AP-1 activation (Angel and Karin, 1991). Therefore, we examined whether this pathway was modulated in the liver of core-transgenic mice. First, we determined JNK activity by *in vitro* kinase assay. As shown in Fig. 2A, the JNK activity was increased in the livers of core-transgenic mice. Densitometry showed that the JNK activity was increased approximately 2.5-fold compared to that of nontransgenic mice. This was almost consistent with the result that the expression of both TNF-α and IL-1β was about 2-fold increased in the liver of core-transgenic mice (Table 2). We also determined the activity of extracellular signal-regulated kinase (ERK) and found similar activation between core-trans-

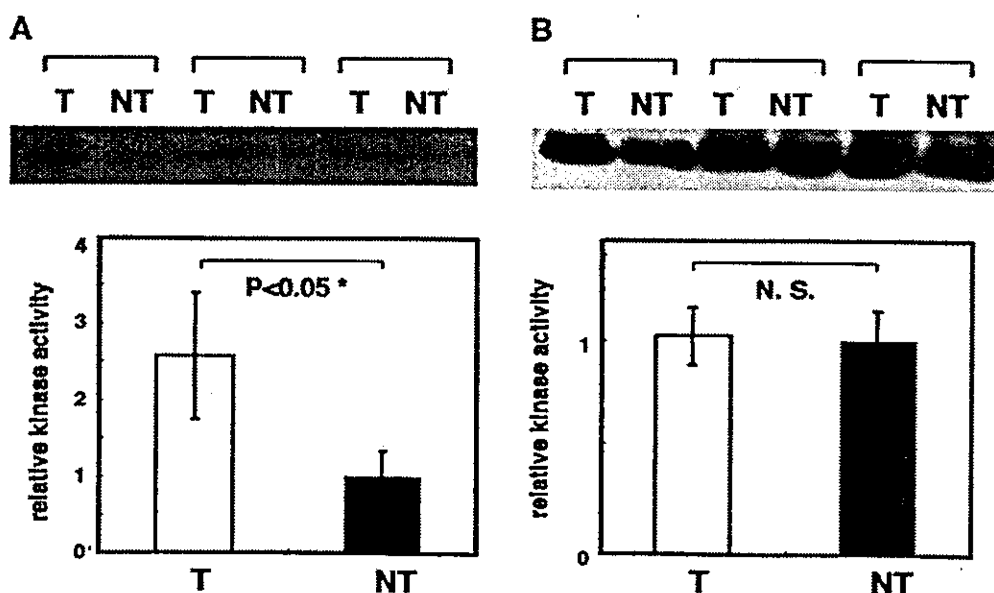


FIG. 2. The activity of JNK and ERK in the liver of core-transgenic (T) and nontransgenic (NT) mice determined by an *in vitro* kinase assay. The *in vitro* kinase assay was performed using commercially available kits. (A) JNK; (B) ERK. The lower panels show densitometric analysis of the blots. The values were normalized by taking the density of nontransgenic mice as 1 (relative kinase activity). The data shown are means ± SE (n = 3 for each sample group). *Mann-Whitney's U test; N. S., no statistically significant difference.

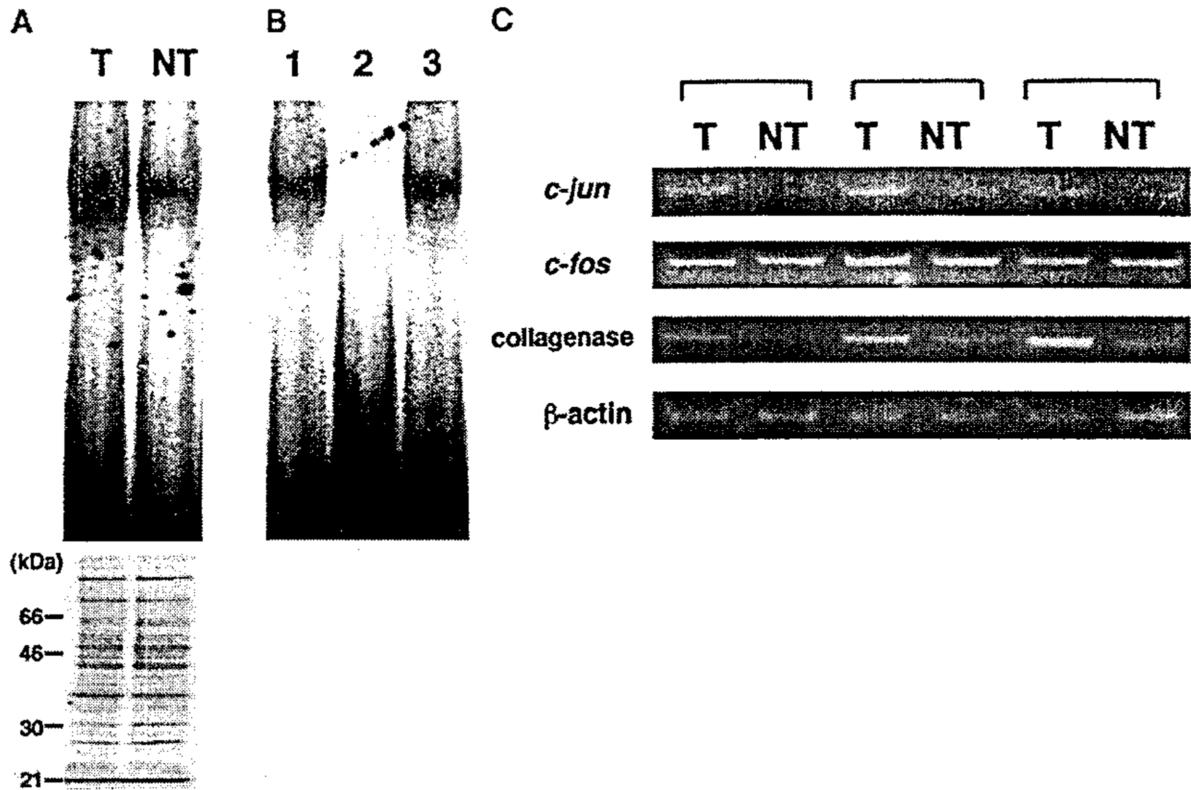


FIG. 3. The activity of AP-1 and expression of AP-1 responsible genes in the liver of core-transgenic (T) and nontransgenic (NT) mice. (A) AP-1 activation. Nuclear proteins were extracted from the liver of mice and incubated with ^{32}P-labeled oligonucleotides containing the AP-1 binding sequence and then loaded onto nondenaturing gels. Coomassie brilliant blue staining of nuclear extracts of the same samples is shown below. (B) Competition assay. Shifted bands corresponding to the AP-1 signal in the 3-month-old, nontransgenic mouse, the same as described in (A), were confirmed by the addition of excess amounts of unlabeled oligonucleotides. Lane 1, no unlabeled oligonucleotides; lane 2, the addition of 50-fold unlabeled AP-1 binding consensus oligonucleotides; lane 3, the addition of 50-fold unlabeled NF-κB binding consensus oligonucleotides. (C) The expression of the AP-1 responsible gene in the liver of core-transgenic mice. Total RNA derived from the livers of mice was subjected to RT–PCR (*c-jun*, 30 cycles; *c-fos*, 35 cycles; collagenase, 35 cycles; β-actin, 20 cycles) using specific primers, and PCR products were separated on a 2% agarose gel.

genic and nontransgenic mice (Fig. 2B). This indicates that JNK was selectively activated in core-transgenic mice.

We next determined the AP-1 activation in the liver of core-transgenic and nontransgenic mice. As shown in Fig. 3A, the activation of AP-1 in core-transgenic mice was markedly enhanced compared to that in nontransgenic littermates. Densitometry showed that the level of AP-1 activity was about two times higher in these transgenic mice. The same amount of nuclear proteins used in the assay was stained by Coomassie brilliant blue after separation by SDS–PAGE (Fig. 3A, lower panel), indicating that a similar amount of nuclear proteins was used between core-transgenic and nontransgenic mouse. We confirmed the specificity of this band by adding 50-fold, unlabeled, identical or different oligonucleotides (Fig. 3B). These results indicate that the JNK–AP-1 pathway was activated in the liver of core-transgenic mice.

AP-1 consists of c-Jun and c-Fos, and the expression of *c-jun* and *c-fos* is regulated mainly by JNK–AP-1 and ERK-SRE, respectively. Therefore, we determined the gene expression of *c-jun* and *c-fos* by RT–PCR. As shown in Fig. 3C, *c-jun* but not *c-fos* expression was increased in the liver of core-transgenic mice. In addition, the expression of the collagenase gene, which is known to be regulated by AP-1 (Angel *et al.*, 1987), was increased in the liver of core-transgenic mice (Fig. 3C). Semiquantitative comparisons of the mRNA abundance revealed that the expression of *c-jun* and collagenase was increased about two- to threefold in the liver of core-transgenic mice, whereas the expression of *c-fos* as well as β-actin was similar. These results indicate that AP-1 activation actually modulated the responsible gene expression in core-transgenic mice.

HCV core protein does not activate the IKK–NF-κB pathway in the liver of core-transgenic mice

TNF-α and IL-1β are known to activate IκB kinase (IKK). IKK phosphorylates IκB, leading to degradation by proteasome. Loss of IκB results in nuclear translocation and activation of NF-κB (Baeuerle, 1991). Therefore, we determined the kinase activity of IKK derived from the

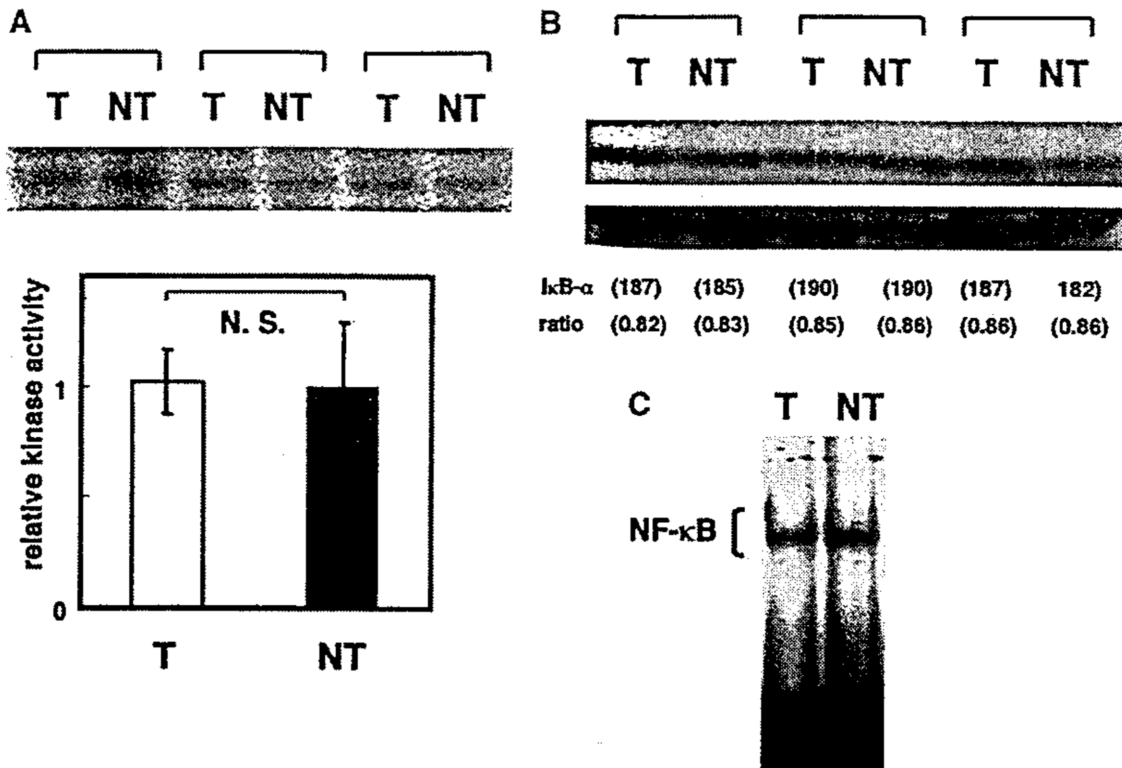


FIG. 4. The activity of IKK and NF-κB in the liver of core-transgenic (T) and nontransgenic (NT) mice. (A) The activity of IKK determined by *in vitro* kinase assay. IKK was immunoprecipitated by anti-IKK antibody from lysates of mouse liver and incubated with GST–IκB-α in the presence of [γ-^{32}P]ATP. The lower panel shows densitometric analysis of the blots. Values were normalized by taking the density of the nontransgenic mice as 1 (relative kinase activity). Data shown are means ± SE (n = 3 for each sample group). N. S., no statistically significant difference. (B) The expression of IκB-α determined by Western blotting. Top, IκB-α; bottom, β-actin. The intensity of the bands corresponding to IκB-α analyzed by densitometry and the ratio of the intensity of IκB-α to that of β-actin is shown below. (C) The NF-κB activation determined by EMSA.

liver of core-transgenic mice. IKK was immunoprecipitated by anti-IKK-α/β antibody, and an *in vitro* kinase assay was performed using the GST–IκB-α (aa 1–54) fusion protein as a substrate. As shown in Fig. 4A, the IKK activity was almost equal in core-transgenic and nontransgenic mice. We also determined the expression of IκB-α in the liver of the mice. As mentioned above, IκB-α was phosphorylated by IKK and rapidly degraded by the ubiquitin–proteasome pathway, so IKK activity should correlate negatively with IκB-α expression. As shown in Fig. 4B, IκB-α expression in the liver of core-transgenic mice was similar to that of nontransgenic mice, further confirming that IKK activity was not activated by the core protein in core-transgenic mice.

We next examined by EMSA whether NF-κB activation reflected the IKK activity and IκB-α expression. As shown in Fig. 4C, NF-κB activation was not significantly more enhanced in core-transgenic mice than in nontransgenic mice, which was in contrast to the enhanced AP-1 activity in core-transgenic mice (Fig. 3A). The specificity of these bands was confirmed by the addition of unlabeled oligonucleotides (data not shown). These results suggest that the IKK–NF-κB pathway was not activated despite the upregulation of TNF-α and IL-1β in core-transgenic mice.

DISCUSSION

We previously demonstrated that HCV core-transgenic mice develop steatosis and later HCC, indicating an important role of the core protein in hepatocarcinogenesis (Moriya *et al.*, 1997b, 1998). In core-transgenic mice, the degree of the core protein expression, determined by Western blotting and fluorescence enzyme immunoassay, was comparable with that in the liver of patients with HCV-associated HCC (Koike *et al.*, 2002). However, in some works from other laboratories, transgenic mice harboring HCV genes showed only mRNA expression but not detectable levels of core protein. Reasons for this may be technical issues in producing transgenic mice, HCV and mouse strains, or the sensitivity of detection systems, particularly antibodies, as discussed previously (Koike *et al.*, 2002). In our core-transgenic mice, (i) HCCs were observed later in their lives, (ii) there was a gender difference in HCC incidence, and (iii) histologically, no inflammation was observed. These characteristics, except for (iii), are analogous to HCV-related HCC in humans. Changes of some host factors may be involved in HCC development as a consequence of the core protein expression. We thus proceeded to investigate the differ-

ential expression of several genes related to cell proliferation in the livers of core-transgenic mice.

In this study, we showed that the expression of TNF-α and IL-1β was significantly enhanced in core-transgenic mice compared to that in nontransgenic littermates. The enhancement of IL-6, in contrast, was not observed. Furthermore, the JNK–AP-1 pathway was activated in the liver cells, but the IKK–NF-κB pathway, another downstream effector of these cytokines, was not enhanced. These modifications of expression of cytokines and transcription factors involved in the signal transduction pathway were observed at as early as 2 months of age in core-transgenic mice, as a consequence of the core protein expression in the liver.

There are conflicting data concerning the association of the core protein with NF-κB activation in previous studies using various cultured cells. Some reports demonstrate that the core protein enhances NF-κB activation (Chung *et al.*, 2001; Marusawa *et al.*, 1999; You *et al.*, 1999), whereas other reports demonstrate that the core protein suppresses TNF-α-induced NF-κB activation (Shrivastava *et al.*, 1998) or does not modulate NF-κB activation in human-derived cells (Zhu *et al.*, 1998). This discrepancy may be due to the differences in the assay systems or cell cultures used. The cells used in the above studies were carcinoma cell lines or transformed cell lines, and their expression levels of the core protein seemed to be considerably higher than that in the liver of HCV-infected patients. Recently, two studies demonstrated that NF-κB was activated in the liver tissue of HCV-infected patients in contrast to that in normal healthy adults (Boya *et al.*, 2001; Tai *et al.*, 2000). However, in these cases one cannot exclude the association of cytokines released by leukocytes, which could contribute to NF-κB activation in the liver, since HCV-infected liver tissue is generally accompanied by inflammatory changes. Our core-transgenic mice expressed the core protein in the liver at a level comparable to that of HCV-infected patients (Koike *et al.*, 2002; Moriya *et al.*, 1998) and exhibited no inflammatory changes throughout their lives. Thus, our core-transgenic mouse model is suitable for investigating the activation of transcription factors, including NF-κB, in the liver by the core protein, and the core protein does not significantly enhance NF-κB activation in the liver, which was otherwise normal.

There are several reports demonstrating the association of the core protein with TNF-α signaling pathways (Chen *et al.*, 1997; Marusawa *et al.*, 1999; Matsumoto *et al.*, 1997; Shrivastava *et al.*, 1998; Zhu *et al.*, 1998). The core protein binds to the TNF receptor superfamily lymphotoxin-β receptor and the cytoplasmic domain of TNF receptor 1 (TNFR1) (Matsumoto *et al.*, 1997; You *et al.*, 1999; Zhu *et al.*, 1998). Such binding implies that the core protein may be involved in the apoptosis signaling pathway and mechanisms of host immune defenses. The present study, for the first time, demonstrates the *in vivo* enhancement of TNF-α expression in the liver by the core protein, which was expressed in otherwise normal liver. Hepatocytes as well as Kupffer cells produce TNF-α (Gonzalez-Amaro *et al.*, 1994; Wordemann *et al.*, 1998); according to a previous study, significant TNF-α production was observed in hepatocytes with chronic HCV infection, whereas TNF-α was detected at low levels in hepatocytes from normal individuals or patients with alcoholic liver diseases (Gonzalez-Amaro *et al.*, 1994). In view of this report and our result here, the core protein may up-regulate TNF-α expression in hepatocytes of core-transgenic mice, as the core protein was detected mainly in hepatocytes of the liver tissues (Moriya *et al.*, 1997b). Alternatively, the core protein expression in hepatocytes may indirectly up-regulate the expression of the cytokines in nonparenchymal cells, such as Kupffer or Kupffer-like cells. Further studies are necessary to clarify this point.

We showed here that the expression of TNF-α and IL-1β, but not IL-6, was enhanced in the liver of core-transgenic mice. Although IL-6 expression is positively regulated by several factors, such as TNF-α, IL-1, AP-1, and NF-κB (Sehgal, 1992), the cooperative interaction of mediators, including negative cross-talk, could be required to achieve the level of IL-6 expression. Indeed, according to a previous report investigating the levels of cytokines in sera in chronic liver diseases, the levels of circulating IL-1β were closely correlated with that of TNF-α, whereas the IL-6 level correlated only weakly with that of TNF-α and did not correlate with that of IL-1β (Tilg *et al.*, 1992). It is noteworthy that a vitronectin gene, which was reported to be regulated positively by IL-6 and negatively by TNF-α and IL-1 (Seiffert *et al.*, 1996), is down-regulated in the liver of core-transgenic mice of all ages (data not shown). Interestingly, a recent report showed that gene expression of vitronectin was also down-regulated in the liver of patients with chronic hepatitis C (Honda *et al.*, 2001). This is compatible with the enhanced expression of TNF-α and IL-1β in the transgenic mice noted in our study. However, contrary to the situation with HCV-infected human livers, our transgenic mice expressed only the core protein. No HCV replication took place. Therefore, it may not be reasonable to directly extrapolate the events that occur in the core-transgenic mice with those in human livers. However, several studies showed that the serum and intrahepatic levels of TNF-α and IL-1β were increased in HCV-infected patients compared to those in healthy controls (Larrea *et al.*, 1996; Tilg *et al.*, 1992). In view of such reports and the fact that there is no *in vitro* culture system for HCV, our core-transgenic mice are regarded as one of the appropriate models for investigating HCV pathogenesis. In addition, it should be noted that our results were not obtained from cultured cells, which already have properties of cancer cells, but from normal

liver cells, which constitute the liver organ in living animals. Future work will be aimed at investigating the mechanism of differential expression of these cytokines in core-transgenic mice.

Our data suggest that the core protein enhances activation of AP-1, a downstream effector of TNF-α and IL-1β. Increased expression of these cytokines in core-transgenic mice possibly contributes to AP-1 activation, since JNK, which phosphorylates and activates c-Jun, a component of AP-1, was also activated. In addition, enhanced expression of *c-jun* but not *c-fos* by AP-1 may also contribute to AP-1 activation. Of course, several other factors may also be associated with AP-1 activation. As reported previously, the core protein enhances basal JNK activity by increasing the phosphorylated form of MAPK kinase (Shrivastava *et al.*, 1998). In addition, two recent studies demonstrated contradictory effects of the core protein on JNK activation; the core protein suppressed TNF-α-induced JNK activation by disrupting the TNFR1–TRADD–TRAF2 complex (Zhu *et al.*, 2001), or enhanced basal and TNF-α-induced JNK activation by forming a ternary complex with TNFR1 and TRADD (Park *et al.*, 2001). In view of these findings, it is possible that the core protein also associates with this complex in core-transgenic mice and influences the downstream pathways.

NF-κB as well as AP-1 are downstream effectors of these cytokines, but NF-κB activation was not obviously enhanced in the core-transgenic mice. Although the reason for this inconsistency remains to be determined, one possibility may be the alteration in the oxidant/antioxidant status in core-transgenic mice (Shrivastava *et al.*, 1998). Previous studies have reported that overexpression of antioxidative enzymes like catalases and superoxide dismutase impairs NF-κB activity (Manna *et al.*, 1998; Nilakantan *et al.*, 1998). It has also been demonstrated that antioxidants like pyrrolidine dithiocarbamate and *N*-acetyl-L-cysteine activate AP-1 and suppress NF-κB (Meyer *et al.*, 1993). Notably, biochemical analyses revealed that our core-transgenic mice had enhanced antioxidative enzyme activity compared to nontransgenic mice of the same age. For example, the catalase activity in core-transgenic mice was 1.5 times higher than that in nontransgenic mice. In addition, the total amount of glutathione, the reduction of which plays a central role in cellular defense against oxidative stress, was significantly lower in core-transgenic mice (Moriya *et al.*, 2001). Taken together, the results lead us to consider that AP-1 activity in core-transgenic mice could be enhanced not only by increased expression of TNF-α and IL-1β, but also by the enhancement of antioxidative enzyme activity. Conversely, NF-κB activity which is activated by increased TNF-α and IL-1β could be diminished by enhanced antioxidative enzyme activity.

In summary, HCV core protein promotes the expression of TNF-α and IL-1β in the liver of core-transgenic mice and activates the JNK–AP-1 pathway. These alterations by the core protein may contribute to the development of liver pathogenesis, including HCC in HCV infection. Further studies are necessary to elucidate the physiological relevance of differential expression of cytokines and AP-1 activation to pathogenesis in persistent HCV infection.

MATERIALS AND METHODS

Transgenic mice

Production of HCV core-transgenic mice (HCV genotype Ib, mouse strain C57BL/6) has been previously described (Moriya *et al.*, 1997b). Expression of the core gene mRNA was detected by RT–PCR. Expression of the core protein in the liver of core-transgenic mice was also confirmed by immunohistochemical staining or Western blotting (Moriya *et al.*, 1997a,b; 1998). Several pairs of male, core-transgenic and nontransgenic mice of 2 to 3 months old were used in this study. Core-transgenic mice had very mild steatosis in the liver, but no inflammatory changes and tumorous lesions, whereas nontransgenic mice had almost normal livers. The blood cells were removed as much as possible from the livers of mice when the mice were sacrificed.

RT–PCR

Mice were sacrificed and total RNA was extracted from the liver using RNAzolB (TEL-TEST, Friendswood, TX). Three micrograms of total RNA were reverse transcribed by Superscript II (GIBCO BRL, Gaithersburg, MD) using oligo(dT) primer. PCR of TNF-α, IL-1β, and GAPDH was performed using the Quantitative PCR Detection Kit (BioSource International, Camarillo, CA). This process can detect multiple gene expression by amplifying all the genes under the same conditions because the PCR primers in this kit have similar melting temperatures and no obvious 3′-end overlap to enhance multiple amplification. Primers for RT–PCR of IL-6 and β-actin were synthesized according to a previous report (Faulkner *et al.*, 1995). Primers for the RT–PCR of *c-jun*, *c-fos*, and collagenase were as follows: *c-jun;* 5′-GCATGAGGAACCGCATTGCCGCCTCCAAGT-3′ and 5′-TCAAAACGTTTGCAACTGCTGCGTTAGCAT-3′; *c-fos:* 5′-TCCTTGAGCATGCCCGTGTCTTAT-3′ and 5′-AGACAAAGGAAGACGTGTAAGTAGTGCAGCC-3′; collagenase: 5′-ATTCCCAAAAGAGGTGAAGAGACT-3′ and 5′-TATGGAATTTGTTGGCATGACTCT-3′. For the RT–PCR, the quantity of cDNA template and the number of amplification cycles were optimized to ensure that the reaction was terminated during the linear phase of product amplification so that semiquantitative comparisons of the mRNA abundance between different samples were possible.

Southern blotting

Five microliters of PCR products was separated on a 1.5% agarose gel containing 1× TBE buffer, denatured

twice in denaturing buffer (0.5 N NaOH, 1.5 M NaCl) for 15 min, neutralized twice in neutralization buffer [0.5 M Tris–HCl (pH 7.5), 3 M NaCl], and transferred onto a Hybond-N$^+$ membrane filter (Amersham, Buckinghamshire, UK) in 20× SSC [3 M NaCl, 0.3 M trisodium citrate dihydrate (pH 7.0)]. The filter was dried, and DNAs were fixed on the membrane by irradiation with UV light and then hybridized with an appropriate heat-denatured probe in hybridization buffer [50% deionized formamide, 5× SSC, 2% blocking reagent (Boehringer Mannheim, Mannheim, Germany), 0.02% SDS, 0.1% *N*-lauroyl salcosin, 0.1 mg/ml of salmon sperm DNA] overnight after prehybridization in the same buffer without a probe for 3 h. Probes were prepared by amplifying and digoxigenin (DIG) labeling the positive control DNA provided in the kit used for PCR. After hybridization, the filter was washed twice with 2× wash buffer (2× SSC, 0.1% SDS) for 5 min and then twice with 0.1× wash buffer (0.1× SSC, 0.1% SDS) for 15 min. DIG-labeled DNA probes were detected using the DIG Luminescent Detection Kit (Boehringer Mannheim).

Real-time PCR

TaqMan probes were labeled with a reporter fluorescent dye [6-carboxy-fluorescein (FAM)] at the 5′-end and a quencher fluorescent dye [6-carboxy-tetramethyl-rhodamine (TAMRA)] at the 3′-end. Primers and the TaqMan probes for TNF-α and IL-1β were as follows: TNF-α forward primer, 5′-CAGACCCTCACACTCAGATCATCT-3′; reverse primer, 5′-CCACTTGGTGGTTTGCTACGA-3′; probe, 5′-(FAM)TCGAGTGACAAGCCTGTAGCCCACGT-(TAMRA)-3′. IL-1β forward primer, 5′-TGGGCCTCAAAGGAAAGAATC-3′; reverse primer, 5′-GACAAACCGCTTTTCCATCTTC-3′; probe, 5′-(FAM)TGCAGCTGGAGAGTGTGGATCCCAA(TAMRA)-3′. Fifty microliters of reaction mixture was used, and it contained 100 ng of extracted total RNA, 0.3 mmol/L forward and 0.9 mmol/L reverse primers, 0.2 mmol/L TaqMan probe, and the TaqMan One-Step RT–PCR Master Mix Reagents Kit (Applied Biosystems, Foster City, CA). The conditions of one-step RT–PCR were as follows: 30 min at 48°C, 10 min at 95°C, and then 60 cycles of amplification for 15 s at 95°C and 1 min at 60°C. The assay used an instrument capable of measuring fluorescence in real time (ABI Prism 7700 Sequence Detector; Applied Biosystems). The calibration curve, covering the range from 1.6 ng to 1 μg total RNA/50 μl reaction, was created using Mouse Liver Total RNA (Ambion, Austin, TX). The same procedure was performed using TaqMan Rodent GAPDH Control Reagents (Applied Biosystems) as an internal control.

ELISA

ELISAs for TNF-α and IL-1β were performed using Mouse TNF-α and IL-1β ELISA Kits (BioSource). Samples were prepared according to a previous paper (Castagliuolo *et al.*, 1998). The ELISA was performed by triplicate for each sample. The amount of total proteins in each sample was determined with the BCA Protein Assay Kit (Pierce, Rockford, IL), and the concentrations of the cytokines in the livers were calculated.

In vitro kinase assay

The activities of JNK and ERK were determined using the SAPK/JNK and the p44/42 MAPK Assay Kit (Cell Signaling Technology, Beverly, MA), respectively. Liver tissues of the mice were homogenized in the lysis buffer provided in the kit, and subsequent assays were performed according to the manufacturer's protocols. The IKK assay was performed using a similar procedure shown in a previous report (Chung *et al.*, 2001). Liver tissues were homogenized in 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM sodium orthovanadate, 1 mM NaF, 0.5 mM β-glycerolphosphate, and Protease Inhibitor Cocktail (Complete; Roche Molecular Biochemicals, Indianapolis, IN). Homogenates were sonicated four times for 5 s and then centrifuged at 14,000 rpm for 10 min. Supernatant containing 500 μg of proteins was incubated overnight with anti-IKK-α/β antibody (Santa Cruz Biotechnology, Santa Cruz, CA) bound to Protein Sepharose 4B beads (Amersham). Immunoprecipitates were washed three times and the kinase reaction was performed in the same buffer with 10 μCi [γ-^{32}P]ATP, 2 mM $MgCl_2$, 1 mM DTT, and 1 μg of GST–IκB-α (aa 1–54) fusion protein as a substrate. GST–IκB-α was prepared in *Escherichia coli* and purified using GST Sepharose Beads (Amersham). The reaction mixture was then separated by SDS–PAGE followed by autoradiography.

Electrophoretic mobility shift assay

Nuclear extracts from the livers of core-transgenic and nontransgenic mice were prepared according to the procedure in a previous report (Deryckere and Gannon, 1994). The double-stranded oligonucleotides (AP-1, 5′-CGCTTGATGAGTCAGCCGGAA-3′; NF-κB, 5′-AGTTGAGGGGACTTTCCCAGGC-3′) were end-labeled with [γ-^{32}P]ATP. Labeled oligonucleotides were incubated on ice for 30 min, along with 5 μg of nuclear extracts, in 30 μl of binding buffer [10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, and 2 μg of poly(dI–dC)]. The binding reactions detecting the AP-1 activity contained 5 mM $MgCl_2$ (Meyer *et al.*, 1993). The reaction mixture was analyzed by electrophoresis on a 7% nondenaturing polyacrylamide gel containing 4% glycerol in 0.25× TBE. For competition experiments, a 50-fold excess of unlabeled, identical oligonucleotides or unrelated oligonucleotides (the NF-κB sequence for AP-1 assay, and the AP-1 sequence for NF-κB assay, respectively) was added 20 min before the addition of labeled oligonucleotides. AP-1 activity was determined by measuring the intensity of the shifted band by densitometer (NIH Image; National Institutes of

Health, Bethesda, MD). A serial 2-fold dilution of purified c-Jun protein (rhAP1; Promega, Madison, WI) was used as a control, and the intensity of each band was measured for standard curves.

Western blotting

Tissue lysates were mixed with SDS sample buffer and sonicated for 5 min. Boiled samples were separated by 10% SDS–PAGE and electrotransfered to a PVDF membrane. After blocking, the membrane was probed with anti-IκB-α antibody (Cell Signaling Technology) or anti-β-actin antibody (Sigma Chemical Co., St. Louis, MO), followed by anti-rabbit IgG conjugated with horseradish peroxidase, and visualized by the Phototope-HRP Western Detection System (Cell Signaling Technology).

Statistical analysis

Results are expressed as means ± SE. The significance of the differences of means was determined using Mann–Whitney's *U* test.

ACKNOWLEDGMENTS

We thank Dr. T. Iwahori for the manipulation of TaqMan PCR and Drs. T. Shimoike, H. Aizaki, S. Takikawa, and R. Suzuki for their helpful discussion. We also thank S. Ogawa, Y. Baba, and M. Matsuda for their technical assistance and T. Mizoguchi for the preparation of the manuscript. This work was supported in part by the Second Term Comprehensive 10-Year Strategy for Cancer Control, Health Sciences Research Grants of The Ministry of Health and Welfare, grants from The Ministry of Education, Science, and Culture of Japan, and by the program for Promotion of Fundamental Studies in Health Sciences of the Organization for Drug ADR Relief, R&D Promotion, and Product Review of Japan. T. Tsutsumi is an awardee of the Research Resident Fellowship from the Foundation for the Promotion of Cancer Research in Japan.

REFERENCES

Andus, T., Bauer, J., and Gerok, W. (1991). Effects of cytokines on the liver. *Hepatology* **13**, 364–375.

Angel, P., Baumann, I., Stein, B., Delius, H., Rahmsdorf, H. J., and Herrlich, P. (1987). 12-*O*-tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5′-flanking region. *Mol. Cell. Biol.* **7**, 2256–2266.

Angel, P., and Karin, M. (1991). The role of Jun, Fos and the AP-1 complex in cell proliferation and transformation. *Biochim. Biophys. Acta* **1072**, 129–157.

Baeuerle, P. A. (1991). The inducible transcription activator NF-κB: Regulation by distinct protein subunits. *Biochim. Biophys. Acta* **1072**, 63–80.

Boya, P., Larrea, E., Sola, I., Majano, P. L., Jimenez, C., Civeira, M. P., and Prieto, J. (2001). Nuclear factor-κB in the liver of patients with chronic hepatitis C: Decreased RelA expression is associated with enhanced fibrosis progression. *Hepatology* **34**, 1041–1048.

Castagliuolo, I., Keates, A. C., Wang, C. C., Pasha, A., Valenick, L., Kelly, C. P., Nikulasson, S. T., LaMont, J. T., and Pothoulakis, C. (1998). Clostridium difficile toxin A stimulates macrophage-inflammatory protein-2 production in rat intestinal epithelial cells. *J. Immunol.* **160**, 6039–6045.

Chantry, D., Turner, M., Abney, E., and Feldmann, M. (1989). Modulation of cytokine production by transforming growth factor-β. *J. Immunol.* **142**, 4295–4300.

Chen, C. M., You, L. R., Hwang, L. H., and Lee, Y. H. (1997). Direct interaction of hepatitis C virus core protein with the cellular lymphotoxin-β receptor modulates the signal pathway of the lymphotoxin-β receptor. *J. Virol.* **71**, 9417–9426.

Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W., and Houghton, M. (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**, 359–362.

Chung, Y. M., Park, K. J., Choi, S. Y., Hwang, S. B., and Lee, S. Y. (2001). Hepatitis C virus core protein potentiates TNF-α-induced NF-κB activation through TRAF2-IKKβ-dependent pathway. *Biochem. Biophys. Res. Commun.* **284**, 15–19.

Cressman, D. E., Greenbaum, L. E., DeAngelis, R. A., Ciliberto, G., Furth, E. E., Poli, V., and Taub, R. (1996). Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. *Science* **274**, 1379–1383.

Deryckere, F., and Gannon, F. (1994). A one-hour minipreparation technique for extraction of DNA-binding proteins from animal tissues. *Biotechniques* **16**, 405.

Diehl, A. M., Yin, M., Fleckenstein, J., Yang, S. Q., Lin, H. Z., Brenner, D. A., Westwick, J., Bagby, G., and Nelson, S. (1994). Tumor necrosis factor-α induces c-jun during the regenerative response to liver injury. *Am. J. Physiol.* **267**, G552–G561.

Faulkner, C. B., Simecka, J. W., Davidson, M. K., Davis, J. K., Schoeb, T. R., Lindsey, J. R., and Everson, M. P. (1995). Gene expression and production of tumor necrosis factor α, interleukin 1, interleukin 6, and γ interferon in C3H/HeN and C57BL/6N mice in acute *Mycoplasma pulmonis* disease. *Infect. Immun.* **63**, 4084–4090.

Gonzalez-Amaro, R., Garcia-Monzon, C., Garcia-Buey, L., Moreno-Otero, R., Alonso, J. L., Yague, E., Pivel, J. P., Lopez-Cabrera, M., Fernandez-Ruiz, E., and Sanchez-Madrid, F. (1994). Induction of tumor necrosis factor α production by human hepatocytes in chronic viral hepatitis. *J. Exp. Med.* **179**, 841–848.

Honda, M., Kaneko, S., Kawai, H., Shirota, Y., and Kobayashi, K. (2001). Differential gene expression between chronic hepatitis B and C hepatic lesion. *Gastroenterology* **120**, 955–966.

Hunt, J. S., Chen, H. L., Hu, X. L., Chen, T. Y., and Morrison, D. C. (1992). Tumor necrosis factor-α gene expression in the tissues of normal mice. *Cytokine* **4**, 340–346.

Koike, K., Moriya, K., Ishibashi, K., Yotsuyanagi, H., Shintani, Y., Fujie, H., Kurokawa, K., Matsuura, Y., and Miyamura, T. (1997). Sialadenitis histologically resembling Sjogren syndrome in mice transgenic for hepatitis C virus envelope genes. *Proc. Natl. Acad. Sci. USA* **94**, 233–236.

Koike, K., Tsutsumi, T., Fujie, H., Shintani, Y., and Moriya, K. (2002). Molecular mechanism of viral hepatocarcinogenesis. *Oncology* **62**, 29–37.

Lai, M. M., and Ware, C. F. (2000). Hepatitis C virus core protein: Possible roles in viral pathogenesis. *Curr. Top. Microbiol. Immunol.* **242**, 117–134.

Larrea, E., Garcia, N., Qian, C., Civeira, M. P., and Prieto, J., P. (1996). Tumor necrosis factor α gene expression and the response to interferon in chronic hepatitis C. *Hepatology* **23**, 210–217.

Manna, S. K., Zhang, H. J., Yan, T., Oberley, L. W., and Aggarwal, B. B. (1998). Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-κB and activated protein-1. *J. Biol. Chem.* **273**, 13245–13254.

Marusawa, H., Hijikata, M., Chiba, T., and Shimotohno, K. (1999). Hepatitis C virus core protein inhibits Fas- and tumor necrosis factor α-mediated apoptosis via NF-κB activation. *J. Virol.* **73**, 4713–4720.

Matsumoto, M., Hsieh, T. Y., Zhu, N., VanArsdale, T., Hwang, S. B., Jeng, K. S., Gorbalenya, A. E., Lo, S. Y., Ou, J. H., Ware, C. F., and Lai, M. M. (1997). Hepatitis C virus core protein interacts with the cytoplasmic tail of lymphotoxin-β receptor. *J. Virol.* **71**, 1301–1309.

McLauchlan, J. (2000). Properties of the hepatitis C virus core protein: A structural protein that modulates cellular processes. *J. Viral Hepatitis* **7**, 2–14.

Meyer, M., Schreck, R., and Baeuerle, P. A. (1993). H_2O_2 and antioxidants have opposite effects on activation of NF-κB and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. *EMBO J.* **12**, 2005–2015.

Moller, A., Henz, B. M., Grutzkau, A., Lippert, U., Aragane, Y., Schwarz, T., and Kruger-Krasagakes, S. (1998). Comparative cytokine gene expression: Regulation and release by human mast cells. *Immunology* **93**, 289–295.

Moriya, K., Fujie, H., Shintani, Y., Yotsuyanagi, H., Tsutsumi, T., Ishibashi, K., Matsuura, Y., Kimura, S., Miyamura, T., and Koike, K. (1998). The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat. Med.* **4**, 1065–1067.

Moriya, K., Fujie, H., Yotsuyanagi, H., Shintani, Y., Tsutsumi, T., Matsuura, Y., Miyamura, T., Kimura, S., and Koike, K. (1997a). Subcellular localization of hepatitis C virus structural proteins in the liver of transgenic mice. *Jpn. J. Med. Sci. Biol.* **50**, 169–177.

Moriya, K., Nakagawa, K., Santa, T., Shintani, Y., Fujie, H., Miyoshi, H., Tsutsumi, T., Miyazawa, T., Ishibashi, K., Horie, T., Imai, K., Miyamura, T., Kimura, S., and Koike, K. (2001). Oxidative stress in the absence of inflammation in the liver of a mouse model for hepatitis C virus-associated hepatocellular carcinoma. *Cancer Res.* **61**, 4365–4370.

Moriya, K., Yotsuyanagi, H., Ishibashi, K., Shintani, Y., Fujie, H., Matsuura, Y., Miyamura, T., and Koike, K. (1997b). Hepatitis C virus core protein induces steatosis in transgenic mice. *J. Gen. Virol.* **78**, 1527–1531.

Nilakantan, V., Spear, B. T., and Glauert, H. P. (1998). Liver-specific catalase expression in transgenic mice inhibits NF-κB activation and DNA synthesis induced by the peroxisome proliferator ciprofibrate. *Carcinogenesis* **19**, 631–637.

Park, K. J., Choi, S. H., Koh, M. S., Kim, D. J., Yie, S. W., Lee, S. Y., and Hwang, S. B. (2001). Hepatitis C virus core protein potentiates c-Jun N-terminal kinase activation through a signaling complex involving TRADD and TRAF2. *Virus Res.* **74**, 89–98.

Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M., Ohta, Y., Choo, Q. L., Houghton, M., and Kuo, G. (1990). Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc. Natl. Acad. Sci. USA* **87**, 6547–6549.

Sehgal, P. B. (1992). Regulation of IL6 gene expression. *Res. Immunol.* **143**, 724–734.

Seiffert, D., Curriden, S. A., Jenne, D., Binder, B. R., and Loskutoff, D. J. (1996). Differential regulation of vitronectin in mice and humans *in vitro*. *J. Biol. Chem.* **271**, 5474–5480.

Shimoike, T., Mimori, S., Tani, H., Matsuura, Y., and Miyamura, T. (1999). Interaction of hepatitis C virus core protein with viral sense RNA and suppression of its translation. *J. Virol.* **73**, 9718–9725.

Shrivastava, A., Manna, S. K., Ray, R., and Aggarwal, B. B. (1998). Ectopic expression of hepatitis C virus core protein differentially regulates nuclear transcription factors. *J. Virol.* **72**, 9722–9728.

Suzuki, R., Suzuki, T., Ishii, K., Matsuura, Y., and Miyamura, T. (1999). Processing and functions of hepatitis C virus proteins. *Intervirology* **42**, 145–152.

Tai, D. I., Tsai, S. L., Chen, Y. M., Chuang, Y. L., Peng, C. Y., Sheen, I. S., Yeh, C. T., Chang, K. S., Huang, S. N., Kuo, G. C., and Liaw, Y. F. (2000). Activation of nuclear factor κB in hepatitis C virus infection: Implications for pathogenesis and hepatocarcinogenesis. *Hepatology* **31**, 656–664.

Tilg, H., Wilmer, A., Vogel, W., Herold, M., Nolchen, B., Judmaier, G., and Huber, C. (1992). Serum levels of cytokines in chronic liver diseases. *Gastroenterology* **103**, 264–274.

Wordemann, M., Fandrey, J., and Jelkmann, W. (1998). Tumor necrosis factor-α production by human hepatoma cell lines is resistant to drugs that are inhibitory to macrophages. *J. Interferon Cytokine Res.* **18**, 1069–1075.

Yamada, Y., Kirillova, I., Peschon, J. J., and Fausto, N. (1997). Initiation of liver growth by tumor necrosis factor: Deficient liver regeneration in mice lacking type I tumor necrosis factor receptor. *Proc. Natl. Acad. Sci. USA* **94**, 1441–1446.

You, L. R., Chen, C. M., and Lee, Y. H. W. (1999). Hepatitis C virus core protein enhances NF-κB signal pathway triggering by lymphotoxin-β receptor ligand and tumor necrosis factor α. *J. Virol.* **73**, 1672–1681.

Zhu, N., Khoshnan, A., Schneider, R., Matsumoto, M., Dennert, G., Ware, C., and Lai, M. M. (1998). Hepatitis C virus core protein binds to the cytoplasmic domain of tumor necrosis factor (TNF) receptor 1 and enhances TNF-induced apoptosis. *J. Virol.* **72**, 3691–3697.

Zhu, N., Ware, C. F., and Lai, M. M. (2001). Hepatitis C virus core protein enhances FADD-mediated apoptosis and suppresses TRADD signaling of tumor necrosis factor receptor. *Virology* **283**, 178–187.