The University *of* Western Ontario
BIOLOGICAL AGENTS REGISTRY FORM
Approved Biohazards Subcommittee: October 14, 2011
Biosafety Website: www.uwo.ca/humanresources/biosafety/

This form must be completed by each Principal Investigator holding a grant administered by the University of Western Ontario (UWO) or in charge of a laboratory/facility where the use of Level 1, 2 or 3 biological agents is described in the laboratory or animal work proposed. The form must also be completed if any work is proposed involving animals carrying zoonotic agents infectious to humans or involving plants, fungi, or insects that require Public Health Agency of Canada (PHAC) or Canadian Food Inspection Agency (CFIA) permits.

This form must be updated at least every 3 years or when there are changes to the biological agents being used.

Containment Levels will be established in accordance with Laboratory Biosafety Guidelines, 3rd edition, 2004, Public Health Agency of Canada (PHAC) or Containment Standards for Veterinary Facilities, 1st edition 1996, Canadian Food Inspection Agency (CFIA).

Electronically completed forms are to be submitted to Occupational Health and Safety, (OHS), (Support Services Building, Room 4190 or to jstanle2@uwo.ca) for distribution to the Biohazards Subcommittee. For questions regarding this form, please contact the Biosafety Officer at extension 81135 or biosafety@uwo.ca. If there are changes to the information on this form (excluding grant title and funding agencies), contact Occupational Health and Safety for a modification form. See website: www.uwo.ca/humanresources/biosafety.

Please ensure that all questions are fully and clearly answered. Failure to do so will lead to the form being returned, which will cause delays in your approval and frustration for you and your colleagues on the Committee.

If you are re-submitting this form as requested by the Biohazards Subcommittee, please make modifications to the form in bold print, highlighted in yellow. Please re-submit forms electronically.

PRINCIPAL INVESTIGATOR:	**C. Yong Kang**
DEPARTMENT:	**Microbiology and Immunology**
ADDRESS:	**1400 Western Road, Rm 129, SDRI, London, ON, N6G2V4**
PHONE NUMBER:	**519-661-3226**
EMERGENCY PHONE NUMBER(S):	**519-871-3991**
EMAIL:	**cykang@uwo.ca**

Location of experimental work to be carried out :

Building :	**Siebens-Drake Research Institute**	Room(s):	**129, 124**
Building :	**HSACF**	Room(s):	**6007 or equivalent**
Building :		Room(s):	

***For work being performed at Institutions affiliated with the University of Western Ontario, the Safety Officer for the Institution where experiments will take place must sign the form prior to its being sent to the University of Western Ontario Biosafety Officer (See Section 15.0, Approvals).**

FUNDING AGENCY/AGENCIES: **Sumagen Canada Inc.**
GRANT TITLE(S): **Development of Hepatitis C Virus Vaccine**
UNDERGRADUATE COURSE NAME(IF APPLICABLE):

List all personnel working under Principal Investigators supervision in this location:

Name	UWO E-mail Address	Date of Biosafety Training
Gyoung Nyoun Kim	**gkim3@uwo.ca**	**Dec., 2011**
Hwayong An	**han5@uwo.ca**	**Dec, 16, 2011**
Kunyu Wu	**kwu29@uwo.ca**	**Dec. 16, 2011**
Elizabeth Banasikowska	**ebanasik@uwo.ca**	**Nov. 29, 2011**
Raji Singh	**rsing5@uwo.ca**	**May 14, 2012**

Please explain how the biological agents are used in your project and how they are stored and disposed of. The BARF without this description will not be reviewed.

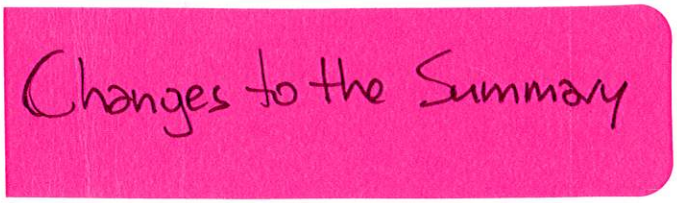
All experiments dealing with vesicular stomatitis viruses, wild type(lab adapted strains) or newly generated mutants from the lab adapted strains are performed in a biological safety cabinet in a level II containment laboratory. The viruses are stored and locked in -80C freezers. The VSVs and other materials used for handling the viruses are all bagged in biohazard bags and are autoclaved for disposal.

Please include a ONE page research summary or teaching protocol in lay terms.
Forms with summaries more than one page will not be reviewed.

Currently we are not working with adenovirus-5. Adenovirus-5 is stored at -80C freezer.

HCV genes (Core, E1, E2, p7, NS3, NS4AB, NS5AB) and HIV genes (Gag, Pol, Env) were inserted into genomes of two serotypes of vesicular stomatitis viruses (VSV;Lab adapted strains), Indiana serotype (rVSV-Ind) and New Jersey serotype (rVSV-NJ). The recombinant VSVs with HCV genes or HIV genes will be injected intramuscularly into mice to induce immune responses against HCV proteins or HIV proteins (Usage of rVSV as vaccine vectors). Mice are prime vaccinated at age of 6 weeks, and 3 weeks after the priming, the mice are booster immunized. A week after the booster immunization, mice are sacrificed for splenocytes and blood sera. The isolated splenocytes are used for the peptide specific (HCV proteins, HIV proteins, or VSV proteins) CD8+ T cell immune responses and sera are checked for the presence of antibodies against HCV proteins or HIV proteins.

We used A549 and HEK 293 cells for the recombinant adenovirus-5 related researchs. We use HCN-1A, HepG2 cells and Huh7 cells to characterize the HCV proteins expressed from the rVSV expressing HCV protiens. We use BHK21, CHO, EBTr-Bovine, E. Derm-Horse, and Vero cells for characterization, amplification, and titration of rVSVs with or without HIV and HCV genes. We use BHK-T7 cells to generate the rVSVs from the cDNA clones by transfection.



1.0 Microorganisms

1.1 Does your work involve the use of biological agents? ☒ YES ☐ NO
(non-pathogenic and pathogenic biological agents including but not limited to bacteria and other microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)? If no, please proceed to Section 2.0

Do you use microorganisms that require a permit from the CFIA? ☐ YES ☒ NO
If YES, please give the name of the species _____
What is the origin of the microorganism(s)? _____

Please describe the risk (if any) of escape and how this will be mitigated:

Please attach the CFIA permit.

Please describe any CFIA permit conditions:

1.2 Please complete the table below:

Full Scientific Name of Biological Agent(s)* (Be specific)	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time? (in Litres)	Source/ Supplier	PHAC or CFIA Containment Level
Vesicular Stomatitis Virus	☐ Yes ☒ No	☒ Yes ☐ No	☐ Yes ☒ No	**0.2 L**		☐ 1 ☒ 2 ☐ 2+ ☐ 3
Adenovirus 5	☒ Yes ☐ No	☐ Yes ☒ No	☐ Yes ☒ No	**2 L**		☐ 1 ☒ 2 ☐ 2+ ☐ 3
E. Coli (competent cells, DH5a, and XL10)	☐ Yes ☒ No	☐ Yes ☒ No	☐ Yes ☒ No	**0.1**	**Invitrogen, Stratagen**	☒ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Y ☐ N			☐ 2 ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Y ☐ N			☐ 2 ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Y ☐ No			☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3

Changes to 1.2

Please attach a Material Safety Data Sheet or equivalent from the supplier if the bacterium used is not on this link:
http://www.uwo.ca/humanresources/docandform/docs/ohs/CFIA_Ecoli_list.pdf

Additional Comments: **MSDS of VSV is attached**

2.0 Cell Culture

2.1 Does your work involve the use of cell cultures? ☒ YES ☐ NO
(If NO, please proceed to Section 3.0)

2.2 Please indicate the type of primary cells (i.e. derived from fresh tissue) that will be grown in culture:

Cell Type	Is this cell type used in your work?	Source of Primary Cell Culture Tissue	AUS Protocol Number
Human	☐ Yes ☒ No		Not applicable
Rodent	☒ Yes ☐ No	**mouse splenocytes**	**2008-106**
Non-human primate	☐ Yes ☒ No		
Other (specify)	☐ Yes ☒ No		

2.3 Please indicate the type of established cells that will be grown in culture in:

Cell Type	Is this cell type used in your work?	Specific cell line(s)*	Containment Level of each cell line	Supplier / Source of cell line(s)
Human	☒ Yes ☐ No	**A549, HepG2, HEK293, HCN-1A, Huh7**	**2 for HEK293, 1 for others**	**ATCC, Dr. Charlse M Rice**
Rodent	☒ Yes ☐ No	**BHK21, CHO, BHK-T7(BSRT7/5),**	**1 for all**	**ATCC, Dr. KARL-KLAUS CONZELMANN**
Non-human primate	☒ Yes ☐ No	**Vero**	**1 for all**	**ATCC**
Other (specify)	☒ Yes ☐ No	**EBTr-Bovine, E. Derm-Horse**	**1 for all**	**ATCC**

Please attach a Material Safety Data Sheet or equivalent from the supplier. (For more information, see www.atcc.org)

2.4 For above named cell types(s) indicate PHAC or CFIA containment level required ☐ 1 ☒ 2 ☐ 2+ ☐ 3

Additional Comments: **Papers on Huh7.5 and BHK-T7(BSRT7/5) are attached**

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? ☐ YES ☒ NO
If no, please proceed to Section 4.0

3.2 Indicate in the table below the Human Source Material to be used.

Human Source Material	**Source/Supplier /Company Name**	**Is H... Mat... With... Age... YES**		
Human Blood (whole) or other Body Fluid		☐ Yes ☐ Unknown		☐1 ☐2 ☐2+ ☐3
Human Blood (fraction) or other Body Fluid		☐ Yes ☐ Unknown		☐1 ☐2 ☐2+ ☐3
Human Organs or Tissues (unpreserved)		☐ Yes ☐ Unknown		☐1 ☐2 ☐2+ ☐3

Changes to 2.3 and 2.4

Human Organs or Tissues (preserved)		Not Applicable		Not Applicable

Additional Comments: _____

4.0 Genetically Modified Organisms and Cell lines

4.1 Will genetic modifications be made to the microorganisms, biological agents, or cells described in Sections 1.0 and 2.0? ☒ YES ☐ NO If **NO**, please proceed to Section 5.0

4.2 Will genetic modification(s) involving plasmids be done? ☒ YES, complete table below ☐ NO

Bacteria Used for Cloning *	Plasmid(s) **	Source of Plasmid	Gene Transformed or Transfected	Will there be a change due to transformation of the bacteria?	Will there be a change in the pathogenicity of the bacteria after the genetic modification?	What are the consequences due to the transformation of the bacteria?
DH5a, XL10 Gold	**Transcript;ion Vector (pTV)**	**Dr. Andrew Ball, University of Texas**	**VSV genome with HCV genes or HIV genes**	**None**	**None**	**None**

E.coli

or equivalent if available.

for the following strains of E. coli:
m/docs/ohs/CFIA_Ecoli_list.pdf

4.3 Will genetic modification(s) of bacteria and/or cells involving viral vectors be made?
☐ YES, complete table below ☒ NO

Virus Used for Vector Construction	Vector(s) *	Source of Vector	Gene(s) Transduced	Describe the change that results from transduction

**** Please attach a Material Safety Data Sheet or equivalent.***

4.3.1 Will virus be replication defective? ☐ YES ☒ NO

4.3.2 Will virus be infectious to humans or animals? ☒ YES ☐ NO

4.3.3 Will this be expected to increase the containment level required? ☐ YES ☒ NO

5.0 Will genetic sequences from the following be involved?

- HIV ☐ NO ☒ YES, specify **Gag, Pol, Env genes**
- HTLV 1 or 2 or genes from any Level 1 or Level 2 pathogens ☒ NO ☐ YES, specify
- SV 40 Large T antigen ☒ NO ☐ YES
- E1A oncogene ☐ NO ☒ YES
- Known oncogenes ☒ NO ☐ YES, specify
- Other human or animal pathogen and or their toxins ☒ NO ☐ YES, specify

5.1 Is any work being conducted with prions or prion sequences? ☒ NO ☐ YES

Additional Comments: **HIV gag, pol, and env genes are inserted into the separate rVSV. The three**

genes are not going to be inserted together into a rVSV.

Changes to this page

6.0 Human Gene Therapy Trials

6.1 Will human clinical trials be conducted involving a biological agent? ☐ YES ☒ NO
(including but not limited to microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)
If no, please proceed to Section 7.0

6.2 If YES, please specify which biological agent will be used:
Please attach a full description of the biological agent.

6.3 Will the biological agent be able to replicate in the host? ☐ YES ☐ NO

6.4 How will the biological agent be administered?

6.5 Please give the Health Care Facility where the clinical trial will be conducted:

6.6 Has human ethics approval been obtained? ☐ YES, number: ☐ NO ☐ PENDING

7.0 Animal Experiments

7.1 Will live animals be used? ☒ YES ☐ NO If **NO**, please proceed to section 8.0

7.2 Name of animal species to be used **Mouse**

7.3 AUS protocol # **2008-106**

7.4 List the location(s) for the animal experimentation and housing. **HSACF, Rm 6007 or equivalent**

7.5 Will any of the agents listed in section 4.0 be used in live animals
☐ NO ☒ YES, specify: **Mouse**

7.6 Will the agent(s) be shed by the animal:
☒ YES ☐ NO, please justify:

8.0 Use of Animal species with Zoonotic Hazards

8.1 Will any animals with zoonotic hazards or their organs, tissues, lavages or other body fluids including blood be used (see list below)? ☐ YES ☒ NO - If **NO**, please proceed to section 9.0

8.2 Will live animals be used? ☐ YES ☐ NO

8.3 If **YES**, please specify the animal(s) used:

♦ Pound source dogs	☐ YES	☐ NO
♦ Pound source cats	☐ YES	☐ NO
♦ Cattle, sheep or goats	☐ YES, species	☐ NO
♦ Non-human primates	☐ YES, species	☐ NO
♦ Wild caught animals	☐ YES, species & colony #	☐ NO
♦ Birds	☐ YES, species	☐ NO
♦ Others (wild or domestic)	☐ YES, specify	☐ NO

8.4 If no live animals are used, please specify the source of the specimens:

9.0 Biological Toxins and Hormones

9.1 Will toxins or hormones of biological origin be used? ☐ YES ☒ NO If **NO**, please proceed to Section 10.0

9.2 If YES, please name the toxin(s) or hormones(s)
Please attach information, such as a Material Safety Data Sheet, for the toxin(s) used.

9.3 What is the LD_{50} (specify species) of the toxin or hormone

9.4 How much of the toxin or hormone is handled at one time*?

9.5 How much of the toxin or hormone is stored*?

9.6 Will any biological toxins or hormones be used in live animals? ☐ YES ☐ NO
If **YES**, Please provide details:

*For information on biosecurity requirements, please see:
http://www.uwo.ca/humanresources/docandform/docs/healthandsafety/biosafety/Biosecurity_Requirements.pdf

Additional Comments: _____

10.0 Insects

10.1 Do you use insects? ☐ YES ☒ NO - If **NO**, please proceed to Section 11.0

10.2 If YES, please give the name of the species.

10.3 What is the origin of the insect?

10.4 What is the life stage of the insect?

10.5 What is your intention? ☐ Initiate and maintain colony, give location:
☐ "One-time" use, give location:

10.6 Please describe the risk (if any) of escape and how this will be mitigated:

10.7 Do you use insects that require a permit from the CFIA permit? ☐ YES ☐ NO
If **YES**, Please attach the CFIA permit & describe any CFIA permit conditions:

11.0 Plants

11.1 Do you use plants? ☐ YES ☒ NO - If **NO**, please proceed to Section 12.0

11.2 If YES, please give the name of the species.

11.3 What is the origin of the plant?

11.4 What is the form of the plant (seed, seedling, plant, tree...)?

11.5 What is your intention? ☐ Grow and maintain a crop ☐ "One-time" use

11.6 Do you do any modifications to the plant? ☐ YES ☐ NO
If yes, please describe:

11.7 Please describe the risk (if any) of loss of the material from the lab and how this will be mitigated:

11.8 Is the CFIA permit attached? ☐ YES ☐ NO
If **YES**, Please attach the CFIA permit & describe any CFIA permit conditions:

12.0 Import Requirements

12.1 Will any of the above agents be imported? ☐ YES, country of origin ☒ NO
If **NO**, please proceed to Section 13.0

12.2 Has an Import Permit been obtained from HC for human pathogens? ☐ YES ☐ NO

12.3 Has an import permit been obtained from CFIA for animal or plant pathogens? ☐ YES ☐ NO

12.4 Has the import permit been sent to OHS? ☐ YES, please provide permit # ☐ NO

13.0 Training Requirements for Personnel Named on Form

All personnel named on the above form who will be using any of the above named agents are required to attend the following training courses given by OHS:

- Biosafety
- Laboratory and Environmental/Waste Management Safety
- WHMIS (Western or equivalent)
- Employee Health and Safety Orientation

As the Principal Investigator, I have ensured that all of the personnel named on the form who will be using any of the biological agents in Sections 1.0 to 9.0 have been trained.

An X in the check box indicates you agree with the above statement... ☒
Enter Your Name C. Yong Kang ***Date:*** Nov. 24, 2011

14.0 Containment Levels

14.1 For the work described in sections 1.0 to 9.0, please indicate the highest HC or CFIA Containment Level required. ☐ 1 ☒ 2 ☐ 2+ ☐ 3

14.2 Has the facility been certified by OHS for this level of containment?
☒ YES, location and date of most recent biosafety inspection: **SDRI, Rm129**
☐ NO, please certify
☐ NOT REQUIRED for Level 1 containment

14.3 Please indicate permit number (not applicable for first time applicants):

15.0 Procedures to be Followed

15.1 Are additional risk reduction measures necessary beyond containment level 1, 2, 2+ or 3 measures that are unique to these agents? ☐ YES ☒ NO
If **YES** please describe:

15.2 Please outline what will be done if there is an exposure to the biological agents listed such as a needlestick injury or an accidental splash:
Exposure to VSV: Clean the site of needlestick injury or accidental splash with 70% ethanol. If the symptoms show, symptomatic treatments are required. Infections of adult humans with the VSV are self-limiting.

15.3 As the Principal Investigator, I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.shs.uwo.ca/workplace/workplacehealth.html

An X in the check box indicates you agree with the above statement... ☒
Enter Your Name C. Yong Kang ***Date:*** Nov. 24, 2011

15.4 Additional Comments: _____ Changes to 15.2

16.0 Approvals

1) UWO Biohazards Subcommittee:
SIGNATURE: _____
Date: _____

2) Safety Officer for the University of Western Ontario
SIGNATURE: _____
Date: _____

3) Safety Officer for Institution where experiments will take place (if not UWO):
SIGNATURE: _____
Date: _____

Approval Number: _____ Expiry Date (3 years from Approval): _____

Special Conditions of Approval:

Hi there

Here are the comments for you (t went to a Committee member for screening...)-

Summary of hazards: State whether the Indiana and New Jersey serotypes are replication-competent or replication-incompetent. If replication-competent, identify the biohazard, particularly: if infection will lead to death (in mouse), if virus will be continually shed in the mouse, if there is a human hazard to personnel.

- Section 1.2: E. coli is level 1
- Section 2.4: Level 2 should be checked
- Section 4.2: Last column should be "None" not "No"
- Section 4.3: Should be Yes. Virus is being injected into mice to transduce HCV and HIV genes. Describe the result in the mouse cells that will be infected, particularly any growth-altering effect in these cells.
- Section 15.2: More details. What is the protocol for accidental exposure? Will any medical treatment be needed?

If possible, please send changes by May 31st.

THanks!

Jennifer

New Info

1.2: corrected
2.4: corrected
4.2: corrected
4.3: Vesicular stomatitis viruses are negative sense RNA viruses, which means their RNA genomes cannot be used as messenger RNAs, but they can be used as templates for the messenger RNAs by VSV RNA dependant RNA polymerase. For the genome replication and transcription of mRNAs of VSV, there is no viral DNA synthesis is involved. VSV with HCV and HIV genes does not incorporate HCV and HIV genes into the host cell genome; it does not transduce HCV or HIV genes into the host cell genome. The recombinant VSVs with gene of interests such as HCV and HIV genes express proteins using their transcriptional system using host cellular translational machinery. The results of the infected cells *in vitro*, and possibly *in vivo* are necrotic and apoptotic cell death.

Two serotypes of vesicular stomatitis viruses in our lab, Indiana serotype and New Jersey serotype are lab-adapted strains of VSV, which were passaged and maintained *in vitro* in mammalian cells such as BHK21 cells. Our recombinant VSVs w/ or w/o HCV genes and HIV-1 genes are generated from the cDNA of the lab-adapted strains of VSV, which were further attenuated from the parental VSVs. We inject mice with VSVs intramuscularly in order to induce immune responses against the gene of interest (HIV or HCV genes). Intramuscular injection of mice does not cause disease in adult mice (6 weeks old). VSVs may be secreted through the urine or feces from the injected mice for about a week or two weeks after injection, and viral secretion stops when immune responses clear VSV. Mice injected with VSVs are kept in the microisolator cages, which are connected to air filtration and ventilation units in the HSACF biosafety level III rooms. Cage cleaning and change are all performed in a biosafety cabinet according to the standard operating procedure.

15.2: answered

Agriculture Canada

Food Production and Inspection Branch

Direction générale, Production et inspection des aliments

Your file Votre référence

Our file Notre référence

2nd Floor West
59 Camelot Drive
Nepean, ON K1A 0Y9
(613) 952-8000
(613) 952-8884 fx

University of Western Ontario

March 24, 1994

Info on VSV

Gillian Norton
Biosafety Officer
University of Western Ontario
Somerville House
London ON N6A 3K7

SUBJECT: Vesicular Stomatitis Virus

Dear Ms. Norton:

Your facsimile dated March 22, 1994 was received. We acknowledge your request for Dr. Y. Kang to use VSV for in vitro studies in laboratory containment facilities at MRC level 2. Authorization is given for the use of the strains of VSV referenced in your letter under these conditions.

In reference to the last paragraph of your letter, if the original viral stocks were brought into Canada under the authority of an import permit then it is necessary for Dr. Kang to obtain permission from the Animal Health Division of Agriculture and Agri-Food Canada prior to transferring this material to any other person or laboratory in Canada. This is clearly stated in a condition printed on the original import permit. A new import permit would not be issued, the letter of authorization to move the material is the only document required.

Sincerely,

B.S. Samagh
Associate Director
Veterinary Biologics
& Biotechnology
Animal Health Division

PG

PS: We would appreciate you referring to the above mentioned file number(s) when referring to the above mentioned subject(s)

Public Health Agency of Canada | Agence de la santé publique du Canada

Canada

Adenovirus types 1, 2, 3, 4, 5 and 7 - Material Safety Data Sheets (MSDS)

MATERIAL SAFETY DATA SHEET - INFECTIOUS SUBSTANCES

SECTION I - INFECTIOUS AGENT

NAME: ***Adenovirus types 1, 2, 3, 4, 5 and 7***

SYNONYM OR CROSS REFERENCE: ARD, acute respiratory disease, pharyngoconjunctival fever

CHARACTERISTICS: *Adenoviridae*; non-enveloped, icosahedral virions, 70-90 nm diameter, doubled-stranded, linear DNA genome.

SECTION II - HEALTH HAZARD

PATHOGENICITY: Varies in clinical manifestation and severity; symptoms include fever, rhinitis, pharyngitis, tonsillitis, cough and conjunctivitis; common cause of nonstreptococcal exudative pharyngitis among children under 3 years; more severe diseases include laryngitis, croup, bronchiolitis, or severe pneumonia; a syndrome of pharyngitis and conjunctivitis (pharyngoconjunctival fever) is associated with adenovirus infection

EPIDEMIOLOGY: Worldwide; seasonal in temperate regions, with highest incidences in the fall, winter and early spring; in tropical areas, infections are common in the wet and colder weather; annual incidence is particularly high in children; adenovirus types 4 and 7 are common among military recruits (ARD)

HOST RANGE: Humans

INFECTIOUS DOSE: >150 plaque forming units when given intranasally

MODE OF TRANSMISSION: Directly by oral contact and droplet spread; indirectly by handkerchiefs, eating utensils and other articles freshly soiled with respiratory discharge of an infected person; outbreaks have been related to swimming pools; possible spread through the fecal-oral route

INCUBATION PERIOD: From 1-10 days

COMMUNICABILITY: Shortly prior to and for the duration of the active disease

SECTION III - DISSEMINATION

RESERVOIR: Humans

ZOONOSIS: None

VECTORS: None

SECTION IV - VIABILITY

DRUG SUSCEPTIBILITY: No specific antiviral available; cidofovir has shown promise in the treatment of adenoviral ocular infections.

SUSCEPTIBILITY TO DISINFECTANTS: Susceptible to 1% sodium hypochlorite, 2% glutaraldehyde, 0.25% sodium dodecyl sulfate

PHYSICAL INACTIVATION: Sensitive to heat>56°C; unusually stable to chemical or physical agents and adverse pH conditions

SURVIVAL OUTSIDE HOST: Resistance to chemical and physical agents allows for prolonged survival outside of the body. Adenovirus type 3 survived up to 10 days on paper under ambient conditions; adenovirus type 2 survived from 3-8 weeks on environmental surfaces at room temperature

SECTION V - MEDICAL

SURVEILLANCE: Monitor for symptoms; confirm by serological analysis

FIRST AID/TREATMENT: Mainly supportive therapy

IMMUNIZATION: Vaccine available for adenovirus types 4 and 7 (used for military recruits)

PROPHYLAXIS: None available

SECTION VI - LABORATORY HAZARDS

LABORATORY-ACQUIRED INFECTIONS: Ten cases documented up to 1988

SOURCES/SPECIMENS: Respiratory secretions

PRIMARY HAZARDS: Ingestion; droplet exposure of the mucous membrane

SPECIAL HAZARDS: Contact with feces from infected animals

SECTION VII - RECOMMENDED PRECAUTIONS

CONTAINMENT REQUIREMENTS: Biosafety level 2 practices and containment facilities for all activities involving the virus and potentially infectious body fluids or tissues

PROTECTIVE CLOTHING: Laboratory coat; gloves when skin contact with infectious materials is unavoidable

OTHER PRECAUTIONS: None

SECTION VIII - HANDLING INFORMATION

SPILLS: Allow aerosols to settle; wearing protective clothing gently cover the spill with absorbent paper towel and apply 1% sodium hypochlorite starting at the perimeter and working towards the centre; allow sufficient contact time (30 min) before clean up

DISPOSAL: Decontaminate all wastes before disposal; steam sterilization, incineration, chemical disinfection

STORAGE: In sealed containers that are appropriately labelled

SECTION IX - MISCELLANEOUS INFORMATION

Date prepared: November 1999

Prepared by: Office of Laboratory Security, PHAC

Although the information, opinions and recommendations contained in this Material Safety Data Sheet are compiled from sources believed to be reliable, we accept no responsibility for the accuracy, sufficiency, or reliability or for any loss or injury resulting from the use of the information. Newly discovered hazards are frequent and this information may not be completely up to date.

Date Modified: 2011-02-18

Canadian Food Inspection Agency | Agence canadienne d'inspection des aliments

Office of Biohazard Containment and Safety
Science Branch, CFIA
59 Camelot Drive, Ottawa, Ontario K1A 0Y9
Tel: (613) 221-7068 Fax: (613) 228-6129
Email: ImportZoopath@inspection.gc.ca

Bureau du confinement des biorisques et sécurité
Direction générale des sciences, ACIA
59 promenade Camelot, Ottawa, Ontario K1A 0Y9
Tél: (613) 221-7068 Téléc: (613) 228-6129
Courriel: ImportZoopath@inspection.gc.ca

October 20th, 2009

Ms. Shamila Survery / Mr. Michael Decosimo
Cedarlane Laboratories Ltd
4410 Paletta Court
Burlington, Ontario L7L 5R2

By Facsimile: (289) 288-0020

SUBJECT: Importation of *Escherichia coli* strains

Dear Ms. Survery / Mr. Decosimo:

Our office received your query about the importation of *Escherichia coli* from the American Type Culture Collection (ATCC) located in Manassas, Virginia, United States. The following *Escherichia coli* strains are consider to be level 1 animal pathogens:

- 5K
- 58
- 58-161
- 679
- 1532
- AB284
- AB311
- AB1157
- AB1206
- AG1
- B
- BB4
- BD792
- BL21
- BL21 (DE3)
- BM25.8
- C
- C-1a
- C-3000
- C25
- C41 (DE3)
- C43 (DE3)
- C600
- Cavalli Hfr
- CIE85
- DH1
- DH10 GOLD
- DH10B
- DH5
- DH5-alpha
- DP50
- DY145
- DY380
- E11
- EJ183
- EL250
- EMG2
- EPI 300
- EZ10
- FDA Seattle 1946
- Fusion-Blue
- H1443
- HF4714
- HB101
- HS(PFAMP)R
- Hfr3000
- Hfr3000 X74
- HMS174
- J52
- J53
- JC3272
- JC7661
- JC9387
- JF1504
- JF1508
- JF1509
- JJ055
- JM83
- JM101
- JM109
- K12
- KC8
- KA802
- KAM32
- KAM33
- KAM43
- LE450
- LE451
- LE452
- MB408
- MBX1928
- MC1061
- MC4100 (MuLac)
- MG1655
- MM294
- MS101
- NC-7
- Nissle 1917
- One Shot STBL3
- OP50
- P678
- PA309
- PK-5
- PMC103
- PR13
- Rri
- RV308
- S17-1λ -PIR
- SCS1
- SMR10
- SOLR
- SuperchargeEZ10
- SURE
- TOP10
- TG1
- U5/41
- W208
- W945
- W1485
- W3104
- W3110
- WA704
- WP2
- X1854
- X2160T
- X2541
- X2547T
- XL1-BLUE
- XL1-BLUE-MRF
- XLOLR
- Y10
- Y1090 (1090)
- YN2980
- W3110
- WG1
- WG439
- WG443
- WG445

The Office of Biohazard Containment and Safety (BCS) of the Canadian Food Inspection Agency (CFIA) only issues import permits for microorganisms that are pathogenic to animals, or parts of microorganisms that are pathogenic to animals. As the products listed above are not considered pathogenic to animals, the Office of BCS does not have any regulatory requirements for their importation.

Please note that other legislation may apply. You may wish to contact the Public Health Agency of Canada's (PHAC) Office of Laboratory Security at (613) 957-1779.

Note: Microorganisms pathogenic to animals and veterinary biologics require an import permit from the CFIA.

Sincerely,

Cinthia Labrie
Head, Animal Pathogen Importation Program
Office of Biohazard Containment & Safety

Canada

Cell Biology

ATCC® Number:	**CCL-185™** Order this Item	Price:	**$279.00**

Cell line(s)

Designations:	A549
Depositors:	M Lieber
Biosafety Level:	1
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Homo sapiens* (human)
Morphology:	epithelial 
Source:	**Organ:** lung **Disease:** carcinoma
Cellular Products:	keratin
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Isolation:	**Isolation date:** 1972
Applications:	transfection host
DNA Profile (STR):	Amelogenin: X,Y CSF1PO: 10,12 D13S317: 11 D16S539: 11,12 D5S818: 11 D7S820: 8,11 THO1: 8,9.3 TPOX: 8,11 vWA: 14

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Cytogenetic Analysis: This is a hypotriploid human cell line with the modal chromosome

Cell Biology

ATCC® Number:	**CRL-11997™** Order this Item	Price:	**$429.00**

Designations:	HEP G2/2.2.1
Depositors:	Northeastern Ohio University College of Medicine
Biosafety Level:	1
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Homo sapiens* (human)
Morphology:	epithelial
Source:	**Organ:** liver **Disease:** hepatocellular carcinoma
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
DNA Profile (STR):	Amelogenin: X,Y CSF1PO: 10,11 D13S317: 9,13 D16S539: 12,13 D5S818: 11,12 D7S820: 10 THO1: 9 TPOX: 8,9 vWA: 17
Age:	15 years
Gender:	male
Ethnicity:	Caucasian
Comments:	Cell line was derived from the hepatocellular carcinoma cell line, HepG2 (ATCC HB-8065). The parental cells were stably transfected at passage 48 with a human cholesterol 7 alpha-hydroxylase (CYP7) minigene/Luciferase construct.

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ATCC complete growth medium: A 1:1 mixture of Dulbecco's

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ATCC® Number:	**CRL-10742™** Order this Item	Price:	**$329.00**
Designations:	HCN-2		
Depositors:	Johns Hopkins University		
Biosafety Level:	1		
Shipped:	frozen		
Medium & Serum:	See Propagation		
Growth Properties:	adherent		
Organism:	*Homo sapiens* (human)		
Morphology:	neuronal		
Source:	**Organ:** brain **Cell Type:** cortical neuron;		
Cellular Products:	tubulin; neurofilament protein; somatostatin; cholecystokinin-8		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		
DNA Profile (STR):	Amelogenin: X CSF1PO: 10,15 D13S317: 11,12 D16S539: 8,11 D5S818: 13 D7S820: 9,11 THO1: 6,9.3 TPOX: 8 vWA: 14,20		
Age:	7 years		
Gender:	female		

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HCN-2 cells can be induced to differentiate when cultured with a

Cell Biology

ATCC® Number:	**CCL-10™** Order this Item	Price:	**$279.00**

Designations:	BHK-21 [C-13]
Depositors:	I Macpherson
Biosafety Level:	1
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	Mesocricetus auratus (hamster, Syrian golden)
Morphology:	fibroblast 
Source:	**Organ:** kidney **Disease:** normal **Cell Type:** fibroblast
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Isolation:	**Isolation date:** March, 1961
Applications:	testing [92346] [92389] transfection host (Roche Transfection Reagents)
Virus Susceptibility:	Human adenovirus 25 Reovirus 3 Vesicular stomatitis virus Human poliovirus 2
Cytogenetic Analysis:	Chromosome Frequency Distribution 50 Cells: 2n = 44. This is a pseudodiploid line with the tetraploidy occurring at 4%. The karyotype is 44,XY,-6,-15,6q+,15q+ in a majority of cells analyzed. The markers 6q+ and 15q+ occurred in most cells. An occasional monosomic or trisomic condition for a normal chromosome was also detected.
Age:	1 day old newborn
HeLa Markers:	N

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The parent line of BHK-21(C-13) was derived from the kidneys of

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ATCC® Number:	**CCL-61™** Order this Item	Price:	**$279.00**

Designations:	CHO-K1
Depositors:	TT Puck
Biosafety Level:	1
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	Cricetulus griseus (hamster, Chinese)
Morphology:	epithelial-like 
Source:	**Organ:** ovary
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Isolation:	**Isolation date:** 1957
Applications:	transfection host
Virus Resistance:	poliovirus 2; modoc virus; Button Willow virus
Cytogenetic Analysis:	Chromosome Frequency Distribution 50 Cells: 2n = 22. Stemline number is hypodiploid.
Gender:	female
Comments:	The CHO-K1 cell line was derived as a subclone from the parental CHO cell line initiated from a biopsy of an ovary of an adult Chinese hamster by T. T. Puck in 1957. The cells require proline in the medium for growth.
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated F-12K Medium, Catalog No. 30-2004. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Temperature:** 37.0°C

Protocol:

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Cell Biology

ATCC® Number:	**CCL-81™**	Order this Item	Price:	**$279.00**

Designations:	Vero
Depositors:	W Hann, JS Rhim
Biosafety Level:	1
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Cercopithecus aethiops*
Morphology:	epithelial 
Source:	**Organ:** kidney **Disease:** normal
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Isolation:	**Isolation date:** March 27, 1962
Applications:	detection of verotoxin efficacy testing malaria biology media testing mycoplasma testing substrate testing transfection host detection of virus in ground beef
Virus Resistance:	Stratford; Apeu; Caraparu; Madrid; Nepuyo; Ossa
Cytogenetic Analysis:	This is a cell line with the hypodiploid chromosome count. The modal chromosome number was 58 occurring in 66% of cells. In most cells, over 50% of the chromosomes in each cell complement belonged to structurally altered marker chromosomes. Normal A3, A4, B4, and B5 were absent; B2, B3 and B7 were occasionally paired; and B9, C1 and C5 were mostly paired. The rate of cells with higher ploidies was 1.7%. Other chromosomes were mostly present in single copy.
Age:	adult

Journal of Virology

Generation of Bovine Respiratory Syncytial Virus (BRSV) from cDNA: BRSV NS2 Is Not Essential for Virus Replication in Tissue Culture, and the Human RSV Leader Region Acts as a Functional BRSV Genome Promoter

Ursula J. Buchholz, Stefan Finke and Karl-Klaus Conzelmann

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Generation of Bovine Respiratory Syncytial Virus (BRSV) from cDNA: BRSV NS2 Is Not Essential for Virus Replication in Tissue Culture, and the Human RSV Leader Region Acts as a Functional BRSV Genome Promoter

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Received 2 July 1998/Accepted 8 October 1998

In order to generate recombinant bovine respiratory syncytial virus (BRSV), the genome of BRSV strain A51908, variant ATue51908, was cloned as cDNA. We provide here the sequence of the BRSV genome ends and of the entire L gene. This completes the sequence of the BRSV genome, which comprises a total of 15,140 nucleotides. To establish a vaccinia virus-free recovery system, a BHK-derived cell line stably expressing T7 RNA polymerase was generated (BSR T7/5). Recombinant BRSV was reproducibly recovered from cDNA constructs after T7 RNA polymerase-driven expression of antigenome sense RNA and of BRSV N, P, M2, and L proteins from transfected plasmids. Chimeric viruses in which the BRSV leader region was replaced by the human respiratory syncytial virus (HRSV) leader region replicated in cell culture as efficiently as their nonchimeric counterparts, demonstrating that all *cis*-acting sequences of the HRSV promoter are faithfully recognized by the BRSV polymerase complex. In addition, we report the successful recovery of a BRSV mutant lacking the complete NS2 gene, which encodes a nonstructural protein of unknown function. The NS2-deficient BRSV replicated autonomously and could be passaged, demonstrating that NS2 is not essential for virus replication in cell culture. However, growth of the mutant was considerably slower than and final infectious titers were reduced by a factor of at least 10 compared to wild-type BRSV, indicating that NS2 provides a supporting factor required for full replication capacity.

Bovine respiratory syncytial virus (BRSV) is a major etiological agent of respiratory tract disease in calves (43). Together with human respiratory syncytial virus (HRSV) and pneumonia virus of mice, it belongs to the genus *Pneumovirus* within the family *Paramyxoviridae* (31). The BRSV genome encodes at least 10 proteins which are expressed by transcription of 10 mRNAs (24, 27). They include two nonstructural proteins (NS1 and NS2); four RNA-associated proteins, namely, the nucleoprotein N, the phosphoprotein P, the large, catalytic subunit L of the RNA polymerase, and a transcription elongation factor encoded by the first of two overlapping open reading frames of the M2 gene (36); and three envelope-associated proteins, namely, the fusion protein F, the attachment protein G, and the small hydrophobic protein SH. Only a little is known about the function of the NS1 and NS2 genes, the presence of which distinguishes the members of the genus *Pneumovirus* from all other paramyxoviruses. The NS1 protein of HRSV was recently found to strongly inhibit transcription and replication in an HRSV minigenome system (1), whereas the function of NS2 is still unclear.

As for all members of the order *Mononegavirales*, the genomic RNA of respiratory syncytial viruses is contained in a ribonucleoprotein (RNP) complex, in which it is tightly encapsidated by N and associated with P and L. Only RNA that is contained within an RNP complex may serve as a template for the viral RNA polymerase (14, 46). Transcription and replication are directed by extragenic promoters which are located at the RNA ends. The genome promoter or leader region is located at the 3′ end of the viral RNA and directs successive transcription of a leader RNA (6) and free subgenomic mRNAs in the order 3′-le-NS1-NS2-N-P-M-SH-G-F-M2-L-5′, as well as synthesis of a full-length antigenomic RNA. The latter appears to involve cotranscriptional encapsidation into the RNP complex. The 3′ end of the antigenome RNA (the complement of the extragenic 5′ region of the genome, which is known as the "trailer") provides *cis*-acting signals that solely direct replicative synthesis of full-length genomic RNAs.

In recent years, protocols that allow intracellular reconstitution of RNP complexes of negative-strand RNA viruses entirely from plasmid-derived components have been made available. Simultaneous expression of full-length antigenome RNA and of individual RNP-associated proteins resulted in the initiation of an infectious cycle and the recovery of recombinant rhabdoviruses (23, 40, 45), paramyxoviruses (2, 4, 10, 13, 17, 18, 20, 32), and a bunyavirus (3). In the case of rhabdoviruses and most paramyxoviruses, N, P, and L proteins were found to be sufficient to render full-length antigenome RNAs infectious. In the case of HRSV, however, the M2 gene had to be expressed in addition to the N, P, and L proteins for successful recovery of recombinant HRSV (4).

We report here the cDNA cloning of the entire BRSV genome and the establishment of a system allowing genetic manipulation of BRSV in order to provide tools for experimental analysis of pneumovirus molecular biology and for development of defined, attenuated vaccines. The integrity of the newly determined nucleotide sequences of the BRSV L gene

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and of the terminal BRSV promoters was confirmed by successful recovery of recombinant BRSV. Interestingly, the HRSV genomic promoter was able to completely substitute for the BRSV sequences. Moreover, the established vaccinia virus-free recovery system allowed us to isolate considerably attenuated virus mutants and to provide direct evidence that the NS2 gene is not essential for BRSV replication.

MATERIALS AND METHODS

Cells and virus. MDBK cells were infected at a multiplicity of infection (MOI) of 0.01 with cell culture supernatant of BRSV, strain A51908 (American Type Culture Collection [ATCC]) (29). After 90 min of adsorption, the inoculum was removed and cells were incubated at 37°C in minimal essential medium supplemented with 3% fetal calf serum (FCS) in a 5% CO_2 atmosphere. After 8 days postinfection, when an extensive cytopathic effect (CPE) was observed, the medium was adjusted to 100 mM $MgSO_4$ and 50 mM HEPES (pH 7.5) (12), and the highly cell-associated virus was released by freezing and thawing. BRSV was also grown on BHK-21 cells (clone BSR T7/5), yielding lower titers. When propagated on BSR T7/5 cells, the virus was harvested after 5 days postinfection, when the CPE was maximal. Titrations were carried out in duplicate in microwell plates by the limiting dilution method. To 0.1 ml of serial 10-fold virus dilutions per well, 10^4 BSR T7/5 cells were added in a 0.1-ml volume. After 48 h, cells were fixed in 80% acetone. An indirect immunofluorescence assay using a bovine serum specific to BRSV was done, and foci of infected cells were counted.

cDNA synthesis and cloning of genome ends. Five days postinfection, total RNA was prepared from MDBK cells infected with BRSV (RNeasy; Qiagen). cDNA clones covering the BRSV genome were generated by specifically primed preparative cDNA syntheses according to Gubler and Hofmann (15). The first-strand reaction was primed with an NS1 gene-specific synthetic oligonucleotide primer (ATue51908 nucleotides [nt] 59 to 79) or with an oligonucleotide derived from the M2/L gene overlap (ATue51908 nt 8376 to 8393). Second-strand synthesis was done with a cDNA synthesis kit (Pharmacia); after *Not*I/*Eco*RI adapter ligation and size selection, the DNA was cloned into Lambda ZAP II phages (Stratagene). Clones containing virus cDNA were identified by plaque hybridization with [α-^{32}P]dCTP (3,000 Ci/mmol; ICN)-labeled (nick translation kit; Amersham) reverse transcription (RT)-PCR fragments derived from the N, G, or M2 gene, or restriction fragments from L gene cDNA clones. From positive phages, recombinant pBluescript SK$^-$ was excised in vivo as recommended by the supplier. At least three independent cDNA clones were sequenced to generate the ATue51908 consensus sequence. The NS1 and NS2 gene sequences (ATue51908 nt 1 to 1146) and the terminal 1.4 kb of the L gene sequence (ATue51908 nt 13701 to 15140) were verified by sequencing of cloned RT-PCR products.

The terminal sequences of genome and antigenome RNAs were determined by polyadenylation and subsequent RT-PCR. Briefly, 5 μg of total RNA from infected cells was incubated for 30 min at 37°C in a 50-μl reaction mix containing 5 units of poly(A) polymerase (Pharmacia Biotech), 40 mM Tris (pH 8.0), 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 250 mM NaCl, 250 μM ATP, and 50 μg of bovine serum albumin per ml. A one-tube RT-PCR containing a proofreading polymerase (Titan RT-PCR Kit; Boehringer Mannheim) was done on 1 μg of polyadenylated RNA. For RT-PCR of the leader region, an oligo(dT) primer containing a *Hin*dIII site and a genome sense primer from the NS1 gene (ATue51908 nt 306 to 286) were used. A fragment of about 300 bp was cloned into pBluescript SK$^-$, and the leader consensus sequence was determined from six individual clones. To determine the trailer end, an additional RT-PCR from the polyadenylated RNA was done, assuming that polyadenylated antigenome template was present. The RT-PCR was primed by the oligo(dT) primer and an antigenome sense oligonucleotide which was derived from the L gene/trailer consensus sequence previously determined (ATue51908 nt 14980 to 15000). The expected fragment of 160 bp was purified and cloned into pBluescript SKII$^-$ prior to sequencing. All sequences were determined by an ABI Prism 377 automated sequencer; data were analyzed with the Genetics Computer Group (GCG) Wisconsin Package, version 9.1 (GCG, Madison, Wis.).

Construction of BRSV full-length plasmids. As a backbone for the construction of transcription plasmids yielding antigenomic full-length RNA, the vector pX12ΔT, which was derived from pX8ΔT (40) by eliminating more restriction sites from the multiple cloning site, was used. The plasmid contains the 84 hepatitis delta virus (HDV) antigenome ribozyme sequence in the *Sma*I site, followed by a T7 RNA polymerase transcription termination sequence in the *Bam*HI site (40).

By a multiple-step cloning procedure, a synthetic T7 RNA polymerase promoter sequence followed by three nonviral G residues and a synthetic leader sequence derived from HRSV strain A2 (nt 1 to 44) (28), the BRSV NS1 and N genes, and the last 0.7 kb of the L gene and adjacent trailer were assembled in pX12ΔT so that the last nucleotide of the trailer is directly followed by the HDV ribozyme sequence. The NS1 noncoding region preceding the translation start codon was modified to contain a synthetic *Not*I tag (see Results). Subsequently, the HRSV leader sequence was replaced by the BRSV leader sequence by site-directed mutagenesis (22) in order to generate nonchimeric recombinants. Both the BRSV construct and the chimeric HRSV-BRSV construct were used as backbone for the assembly of the complete BRSV genome from cDNA. The first plasmids used for recovery of infectious recombinant virus (rH/BRSVΔNS2, rBRSVΔNS2 [see Fig. 1]) were termed pH/BRSVΔNS2, encoding a chimera with HRSV leader sequence and a deletion of the complete NS2 gene, and pBRSVΔNS2, containing the homologous BRSV leader sequence.

The last step for generation of a recombinant full-length construct consisted of the insertion of the NS2 gene. The NS2 gene was obtained by RT-PCR, cloned in pBluescriptSK$^-$ and sequenced, and transferred into the singular *Kpn*I site of the full-length clones (ATue51908 nt 1,026) after restriction of the PCR fragment with *Acc*65I and *Ban*II and Klenow treatment of all ends, resulting in a deletion of 4 nt (positions 510 to 513) from the NS1 noncoding region following the NS1 translation stop codon. The resulting full-length plasmids were termed pBRSV and pH/BRSV, the latter containing the HRSV derived leader region. Figure 1 gives an overview of the four different recombinants recovered from cDNA.

Construction of expression plasmids pN, pP, pL, and pM2. To allow cap-independent translation of mRNAs in T7 RNA polymerase-expressing cells, the encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) (11) was cloned downstream of the T7 RNA polymerase promoter of plasmid pT7T (9). PCR fragments in which the respective translation start codon is contained in an *Nco*I or *Afl*III restriction site were used to clone the open reading frame of the BRSV N (ATue51908 nt 1144 to 2605), P (ATue51908 nt 2351 to 3249), M2 (ATue51908 nt 7523 to 8436), or L (ATue51908 nt 8414 to 15140) gene into the *Nco*I site of the IRES specifying the translation start codon. Inserts generated by PCR were sequenced completely.

Establishment of a cell line stably expressing phage T7 RNA polymerase. To obtain a cell line stably expressing T7 RNA polymerase, 10^6 BHK-21 cells (clone BSR-CL13) were transfected with 1 μg of pSC6-T7-NEO encoding the T7 RNA polymerase gene under control of the cytomegalovirus promoter and the neomycin resistance gene (32) (kindly provided by M. Billeter). Resistant clones were selected after addition of geneticin (1 mg/ml) to the cell culture medium. T7 RNA polymerase expression of selected cell clones was monitored after transfection of 5 μg of pT7T-N, which encodes the rabies virus N protein downstream of the T7 RNA polymerase promoter (9). Two days after transfection, cells were stained with a fluorescein isothiocyanate conjugate recognizing rabies virus N protein (Centocor) and analyzed by immunofluorescence microscopy (not shown). After two further cloning steps, a cell clone which constitutively expressed T7 RNA polymerase (BSR T7/5) was selected for further use. Even after 100 successive passages (with every second passage involving geneticin selection), no decrease in T7 RNA polymerase-directed gene expression was observed in BSR T7/5 cells.

Transfection experiments and recovery of recombinant BRSV (rBRSV). BSR T7/5 cells were grown overnight to 80% confluency in 32-mm-diameter dishes in Eagle's medium supplemented with 10% FCS. One hour before transfection, cells were washed twice with medium without FCS. Cells were transfected with a plasmid mixture containing 10 μg of full-length plasmid (pBRSV, pH/BRSV, pBRSVΔNS2, or pH/BRSVΔNS2), 4 μg of pN, 4 μg of pP, 2 μg of pM2, and 2 μg of pL. Transfection experiments were carried out with a mammalian transfection kit ($CaPO_4$ transfection protocol; Stratagene). The transfection medium was removed at 4 h posttransfection; cells were washed and maintained in Eagle's medium containing 3% FCS.

Five days after transfection, cells were split at a ratio of 1:3. Between 7 and 10 days posttransfection, a typical CPE was observed, yielding several foci per dish. The cells were split every 4 to 5 days, until between days 21 and 28 posttransfection a total CPE was observed. The virus was released by freezing and thawing. Cellular debris was removed by pelleting at 800 × *g*, and the supernatant was used for production of high-titer material in MDBK cells and for further experiments.

Northern hybridization of viral RNA. Total RNA from BSR T7/5 cells was isolated at 2 to 5 days postinfection (RNeasy; Qiagen). The RNA was analyzed by denaturing gel electrophoresis (39), blotted onto nitrocellulose, cross-linked to membranes by UV, and hybridized with DNA fragments labeled (nick translation kit; Amersham) with [α-^{32}P]dCTP (3,000 Ci/mmol; ICN). The N gene-specific probe was generated by RT-PCR and tested for specificity by sequencing analysis before use; the NS2-specific probe consisted of an *Ase*I (ATue51908 nt 574)/*Kpn*I (ATue51908 nt 1026) fragment of a cloned and sequenced RT-PCR product which contains the complete NS2 open reading frame. Northern blots were exposed to Kodak X-Omat AR films at −70°C with an intensifying screen.

RT-PCR. To generate DNA probes for hybridization, for cloning of expression plasmids, and for RNA analysis of recombinant virus, RT-PCR was done with avian myeloblastosis virus reverse transcriptase (Life Sciences) for first-strand synthesis, as described above, and a proofreading thermostable polymerase (*Pfu*; Stratagene) for PCR under conditions recommended by the supplier. The RNA of the recombinant viruses was analyzed by two sets of RT-PCR. The first was designed to demonstrate the synthetic *Not*I restriction site following the NS1 gene start. First-strand primers were selected to hybridize to the 3' end of genomic RNA. The reverse primer (ATue51908 nt 306 to 286) was derived from the NS1 gene sequence. The second PCR was designed to demonstrate the absence or presence of the NS2 gene. First-strand synthesis was done with the same set of leader-specific primers, whereas a primer derived from the N gene sequence (ATue51908 nt 1165 to 1146) served as reverse primer.

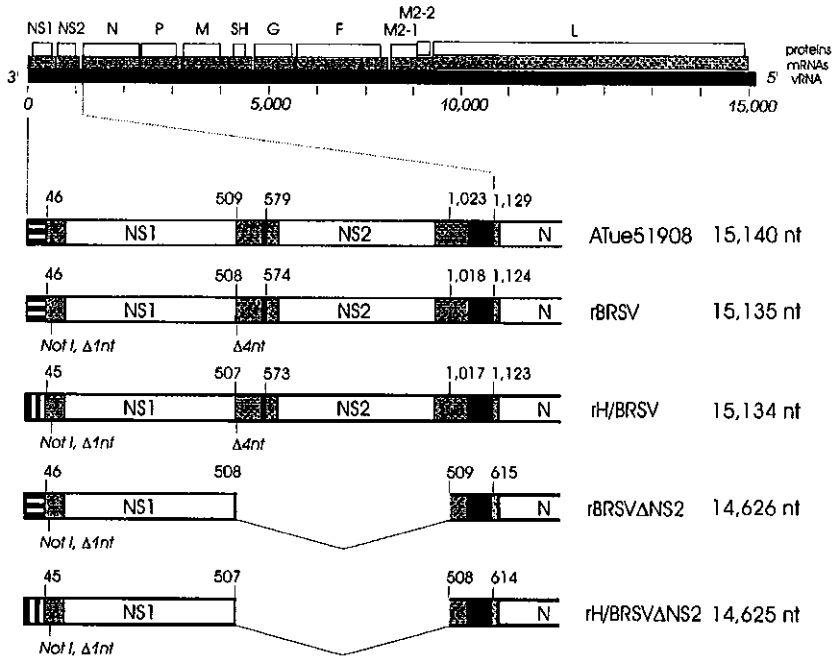

FIG. 1. Schematic presentation of the BRSV ATue51908 genome (drawn to scale). The locations of transcripts (shaded bars) and protein-encoding frames (open bars) are shown relative to the viral RNA (vRNA) (solid bar). In the enlargements, the organizations of recombinant viruses and ATue51908 are compared. The 3'-terminal 45-nt BRSV leader regions are marked by horizontal stripes, and the 44-nt HRSV A2 leader regions are marked by vertical stripes. Genetic tags (*NotI*) and nucleotide deletions in the NS1 noncoding regions (shaded) of the recombinant viruses are indicated. The relative positions of corresponding nucleotides are given for the gene starts of NS1, NS2, and N and nucleotides flanking the NS2 deletion. The overall lengths of the vRNAs are shown on the right.

Nucleotide sequence accession number. The nucleotide sequence of BRSV strain ATue51908 has been deposited in the GenBank database under accession no. AF092942.

RESULTS

cDNA cloning and sequence analysis of BRSV strain ATue51908. We generated cDNA clones covering the complete genome of BRSV strain A51908, as obtained from the ATCC, after 12 passages in MDBK cells. The sequence of the 3' half of the BRSV genome was available, covering nine genes from the NS1 gene to the M2-L gene overlap (25, 26, 30, 37, 38, 47, 48) (see Fig. 1). Two primers were selected for specific priming of first-strand cDNA synthesis, the first hybridizing to the 3' proximal NS1 noncoding region and the second hybridizing to the M2-L gene overlap. Total RNA of BRSV-infected cells was used as starting material. Virus-specific cDNA clones were detected by hybridization with PCR probes derived from the N, G, and M2 genes. Each probe was tested for specificity by sequence analysis and by hybridization with virus RNA before use. Clones covering the entire L gene were obtained by genome walking. Besides the correctly primed cDNA clones, we found a considerable amount of BRSV-specific clones that were random primed, together covering the entire genome except for the NS2 gene. A consensus sequence was derived from at least three independent cDNA clones. The last 1.4 kb of the genomic sequence and the NS1 and NS2 gene sequences were, in addition, confirmed by sequencing of cloned RT-PCR fragments. In this case, at least six different PCR clones were analyzed to generate a consensus sequence.

Nucleotides 46 to 8454 of the consensus sequence, spanning the NS1, NS2, N, P, M, SH, G, F, and M2 genes, were compared to the previously published sequences of BRSV strain A51908 (GenBank accession no. U15937 [NS1], U15938 [NS2], M35076 [N], M93127 [P], D01012 [M and SH], and M82816 [F and M2] [26, 48]), revealing considerable divergence. The total sequence identity was found to be 96.2%, owing to 319 differences and 10 gaps in 8,454 nt. For the NS1, NS2, N, P, M, F, and M2 genes, sequence identities were in the range of 95 to 98%, and the amino acid identity ranged between 100 and 97%. Both the SH and the G genes exhibited nucleotide identity of merely 93% with the published A51908 sequences. In the deduced SH and G proteins, only 86 and 88% of the amino acids are identical, respectively. Together, the predicted amino acid sequences of the nine genes contain 97 differences compared to the A51908 sequence, over a total of 2,263 amino acids. Since the sequence data obtained differed substantially from the published A51908 sequence, we designated our starting virus BRSV strain ATue51908. The total sequence of BRSV strain ATue51908 (GenBank accession no. AF092942) comprises 15,140 nt and codes for 10 mRNAs (Fig. 1). As described for other strains, the G and M2 mRNAs each contain two open reading frames.

Sequence of the ATue51908 L gene. Until now, the sequence of the largest BRSV gene coding for the catalytic subunit of the viral RNP-dependent RNA polymerase (L) was not available. The BRSV L gene has a length of 6,573 nt, including the gene start signal (5'-GGACAAAA-3' [DNA positive strand]) and the transcription stop and polyadenylation signal (5'-AGTTA TTTAAAAA-3' [positive strand]). The first ATG located in a favorable context for initiation of translation is partially contained in the gene start signal (5'-GGACAAAATG-3'), resulting in an extremely short 5' noncoding region of 7 nt. The translation stop codon, TAA, is followed by a noncoding region of 64 nt and the transcription stop/polyadenylation signal. The predicted open reading frame codes for a protein of 2,162

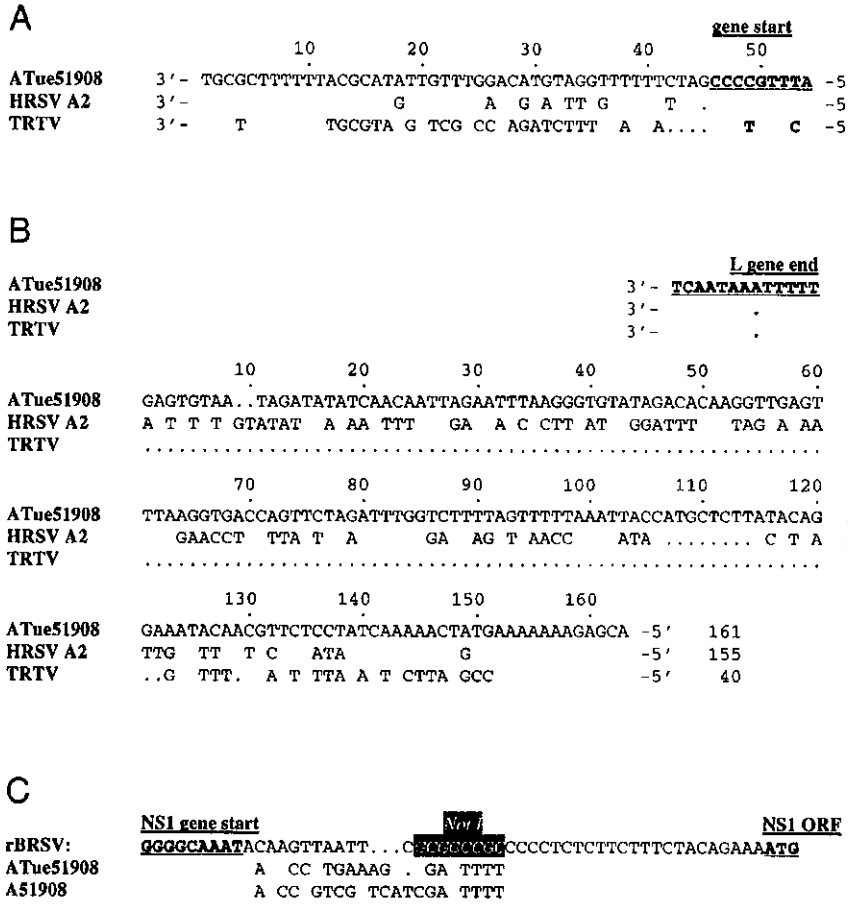

FIG. 2. Alignment of the BRSV ATue51908 3′ leader region (A) and 5′ trailer (B) sequences with those from other pneumoviruses. The DNA sequences are shown in 3′-to-5′ viral RNA sense. For HRSV strain A2 (28) and TRTV (33), only deviations from the ATue51908 sequence are indicated. Gaps are represented by dots, and the start signal of the first gene (NS1 in BRSV and HRSV, N in TRTV) and the L gene end signal are underlined. Numbering of the trailer sequences starts with the first nucleotide downstream of the L gene transcription stop signal. (C) Alignment of the NS1 noncoding region of the recombinant BRSVs containing the *Not*I tag (boxed) with the ATue51908 consensus sequence and the published A51908 sequence. The sequences are shown as DNA positive strands. Only nucleotides differing from the recombinant sequence are indicated. Gaps are indicated by dots, and the NS1 gene start signal and translation start codon are underlined.

amino acids. No additional open reading frames longer than 30 amino acids were identified.

Compared to the L gene of HRSV strain A2 (41), the BRSV L gene has identity of 77% and encodes a protein with amino acid identity of 84%. The L proteins of BRSV, HRSV, and turkey rhinotracheitis virus (TRTV) (34) share an amino-terminal extension of about 20 amino acids and a carboxy-terminal truncation of about 100 amino acids, compared to L proteins of other nonsegmented negative-strand RNA viruses. This feature therefore appears to be common in the *Pneumovirinae* subfamily.

Sequence analysis of the BRSV leader and trailer regions. The leader region of BRSV (Fig. 2A) is 45 nt in length, 1 nt longer than that of HRSV A2 (28). The terminal regions are highly conserved. Compared to HRSV, the first 17 nt are identical and the first 28 nt contain two differences. Overall, the two RSV leader regions have identity of 82%. Alignment of the BRSV and HRSV leader regions to that of TRTV (33) revealed identities of 61 and 67%, respectively.

The 5′ extragenic region of the BRSV genome (Fig. 2B) is composed of 161 nt, which is 6 nt longer than the HRSV A2 trailer region (28). This is thus far the longest trailer sequence of a member of the *Paramyxoviridae* family. The BRSV leader and trailer regions themselves exhibit a terminal complementarity of the extreme 11 nt which is interrupted at position 4, as is also found in TRTV. The 5′-terminal 14 nt of BRSV and HRSV are identical, and the 5′-terminal 25 nt contain only one different nucleotide. The L-proximal two-thirds of the trailer regions show a very low degree of homology, and the overall identity is 51%. The TRTV trailer region (33), which is composed of only 40 nt, is more similar to the aligned region of HRSV than of BRSV (60 and 50% identity, respectively).

Construction of full-length BRSV cDNA clones. A cDNA copy of the BRSV genome was assembled in pX12ΔT downstream of a T7 RNA polymerase promoter such that antigenomic RNA would be transcribed. Three G residues were introduced between the promoter and the leader sequence to facilitate initiation of transcription. It was previously shown that additional nucleotides at the 5′ ends of HRSV genomes do not interfere with encapsidation and replication (35). To achieve correct cleavage of the RNA transcript at the 3′ end of the antigenome-like RNA, the HDV antigenome ribozyme sequence was joined directly to the last nucleotide of the trailer sequence (40).

At first, an NS2 deletion mutant was assembled in a plasmid containing the HRSV A2 leader region (nt 1 to 44) (28) instead of the BRSV leader region and in which the NS1 gene end signal, the NS2 gene start signal, and the complete NS2

coding region (514 nt) are absent (rH/BRSVΔNS2 [Fig. 1]). Thus, the NS1 translation stop codon is followed by the gene end signal derived from the NS2 gene. Subsequently, an RT-PCR-generated NS2 gene sequence was introduced into the NS2-deficient cDNA to generate a nondeficient, chimeric full-length genome (rH/BRSV [Fig. 1]). Due to the cloning procedure, 4 nt from the wild-type noncoding sequence between the NS1 translation stop codon and the NS1 gene end signal (nt 510 to 513) were deleted. To finally replace the HRSV leader sequence by the original BRSV leader sequence, site-directed mutagenesis was done, giving rise to rBRSVΔNS2 and rBRSV (for details, see Materials and Methods).

All four cDNA constructs contain three common differences from the standard ATue51908 sequence: a 16-nt difference, involving deletion of one nucleotide, in the NS1 5′ noncoding region, creating a singular *Not*I site and allowing easy discrimination of recombinant virus and wild-type ATue51908 virus (Fig. 2C), as well as two single nucleotide exchanges which are derived from one of the cDNA clones used for cloning (pL23). One of the exchanges is located in the BRSV trailer sequence (nt 15049, T to C) and the other in the L coding sequence (nt 14312, A to C), resulting in an amino acid change from isoleucine to leucine. Overall, the genome of the full-length BRSV recombinant is 5 nt shorter than the standard ATue51908 virus genome, and the chimeric rH/BRSV sequence is 6 nt shorter, due to the shorter HRSV leader. The genomes of the NS2 deletion mutants rBRSVΔNS2 and rH/BRSVΔNS2 are lacking 514 internal nucleotides (Fig. 1).

Recovery of infectious BRSV from cDNA in a vaccinia virus-free cell system. Most systems for recovery of negative-strand RNA viruses make use of vaccinia helper virus providing T7 RNA polymerase for intracellular transcription of full-length RNAs and expression of support proteins. However, to prevent possible interference with the notoriously very slow BRSV replication or virus assembly and to avoid the necessity of separating vaccinia virus from BRSV, we decided to establish a vaccinia virus-free system. A cell line which stably expresses phage T7 RNA polymerase (BSR T7/5) was generated by transfection of BHK-21 cells, clone BSR-CL13, with a plasmid expressing phage T7 RNA polymerase under control of the cytomegalovirus promoter (pSC6-T7-NEO, kindly provided by M. Billeter). BSR T7/5 cells were transfected with plasmids expressing antigenomic BRSV RNA and support plasmids expressing the BRSV N, P, L, and M2 proteins. The support plasmids contained the consensus sequence of the respective ATue51908 open reading frame, downstream of the ECMV IRES sequence, to allow cap-independent translation. Five days after transfection, cells were split in a 1:3 ratio. Between days 7 and 10 after transfection, a typical CPE was observed, in the form of several foci of fusing cells per dish. When any of the support plasmids was omitted, no foci were observed.

All transfection experiments were done in quadruplicate and repeated at least three times. Each of the four different full-length constructs yielded recombinant virus in all dishes. The cells were split every 4 to 5 days, and the recovered virus was released by freezing and thawing when the CPE was maximal. Clarified supernatant was used for three rounds of amplification in the case of the full-length recombinant. In order to amplify the NS2 deletion mutant, at least five passages were necessary to produce stock material suitable for further characterizations.

Identification of genetic tags and transcription analysis of recombinant virus. Genetic tags were used to confirm the identity of the recombinant viruses. Total RNA of virus-infected BSR T7/5 cells was isolated when the CPE was maximal, and RT-PCR was performed. One assay was done to demonstrate the novel *Not*I restriction site contained in the NS1 5′ noncoding region of all recombinant viruses. Primers LeBRSV or LeH/BRSV and NS1r, which are specific for the leader regions and the NS1 gene, respectively, were used to amplify a 300-bp fragment which was then digested with *Not*I. The RT-PCR products obtained from recombinant viruses were cleaved by *Not*I, whereas the fragment obtained from standard ATue51908 virus was not (Fig. 3). A second RT-PCR with the leader-specific primer and the primer Nr, specific for the N gene, was designed to reveal the absence or presence of the NS2 gene. A PCR product of 1.2 kb was obtained in the case of standard ATue51908 virus or of full-length recombinant viruses (Fig. 4). In the case of rBRSVΔNS2 and rH/BRSVΔNS2, a fragment of about 0.7 kb was amplified by the same primers, demonstrating the deletion of the NS2 gene. No PCR product was detected when the RT step was omitted (not shown), demonstrating that the PCR products originate from viral RNA rather than from plasmid contaminations.

In order to demonstrate transcripts from the recombinant

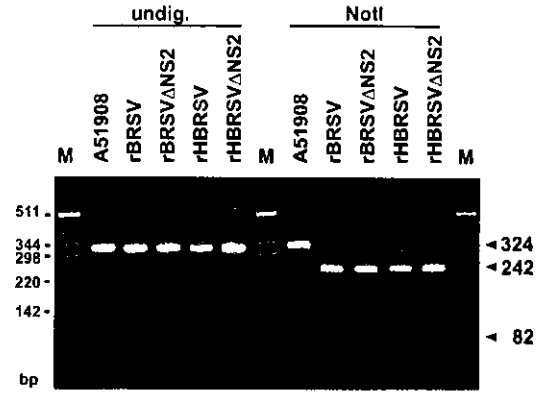

FIG. 3. Demonstration of the *Not*I tag in the genome RNA of recombinant BRSVs. RT-PCR was performed on total RNA of BSR T7/5 cells infected with standard BRSV ATue51908 or with recombinant virus by using positive-sense primers annealing to the genome 3′ ends and a negative-sense primer binding in the NS1 gene. No PCR product was detected when the RT step was omitted. Digestion with *Not*I of the 324-bp (calculated size) RT-PCR product originating from recombinant viruses yielded two bands of calculated sizes of 82 and 242 bp, whereas the RT-PCR product from standard BRSV strain ATue51908 was not cleaved.

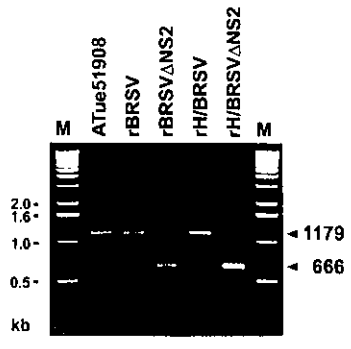

FIG. 4. Demonstration of NS2 gene deletion by RT-PCR. Total RNA of BSR T7/5 cells infected with standard BRSV ATue51908 or with recombinant virus was used for RT-PCR with a positive-sense primer hybridizing to the 3′ end of the BRSV genome RNA and a negative-sense primer binding in the N gene. Products from full-length recombinants and wild-type virus yielded a product of 1,179 bp (calculated size) spanning the NS2 gene, whereas deletion mutants gave rise to a 666-bp (calculated) fragment, reflecting the 513-nt deletion. No PCR product was detected when the RT step was omitted.

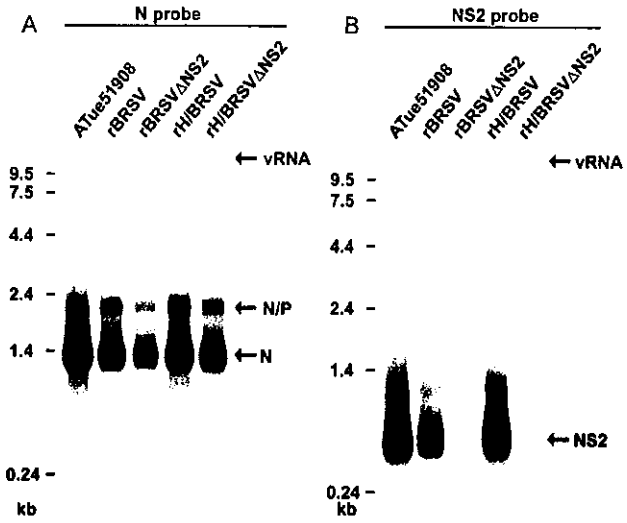

FIG. 5. Demonstration of virus transcripts by Northern hybridization. Total RNA of BSR T7/5 cells infected with rBRSV or standard ATue51908 was isolated 2 to 4 days after infection and separated on a 2% denaturing agarose gel. The blot was hybridized with PCR-derived probes specific for the BRSV N gene (nt 1429 to 2277) (A) and for the NS2 gene (nt 574 to 1026) (B). Transcripts corresponding to N mRNA (vRNA), bicistronic N/P readthrough transcripts, and NS2 mRNA are indicated.

viruses and from standard ATue51908, RNA of infected cells was analyzed by Northern hybridization. Total cellular RNA was isolated 5 days after infection of BSR T7/5 cells at an MOI of 0.01. After hybridization with an N-specific probe, genome RNAs and monocistronic N mRNAs were detected in cells infected with the recombinant BRSVs and standard ATue51908, demonstrating efficient transcription and replication of the recombinant BRSVs (Fig. 5). In addition, a prominent band representing bicistronic N/P readthrough mRNA was present in all lanes. RNA from cells infected with the NS2 deletion mutants did not hybridize with an NS2 probe, whereas a prominent band corresponding to NS2 mRNA was present in cells infected with ATue51908 or the full-length recombinants (Fig. 5). These results again confirmed the absence of the complete NS2 gene in the genome of deletion mutants, as well as the absence of contaminating helper virus. The NS2 deletion mutants thus replicate autonomously in cell culture.

Recombinant viruses lacking the NS2 gene are attenuated in cell culture. Growth characteristics of recombinant BRSV were studied with both the BHK-derived BSR T7/5 cell line and MDBK cells. The two full-length recombinants did not differ from standard ATue51908 virus in their final titers of infectious virus nor in their growth kinetics or phenotype. In MDBK cells, ATue51908 and the full-length recombinants yielded maximal titers of 10^6 PFU/ml after 8 days of infection at an MOI of 0.01, while in BSR T7/5 cells maximum titers of 10^5 PFU/ml were achieved at 4 days after infection at the same MOI. The chimeric viruses containing the HRSV leader region did not differ in any respect from the recombinants containing the autologous BRSV leader sequence. Compared to the full-length viruses, the NS2 deletion mutants showed slower growth and reduced final titers in both BSR T7/5 and MDBK cells. In BSR T7/5 cells, a similar type of CPE, consisting of large syncytia detaching from the monolayer, developed after infection with both full-length and NS2-deficient viruses (Fig. 6A). When grown on MDBK cells, the full-length viruses caused typical foci of enlarged cells, indistinguishable from standard ATue51908 virus foci (Fig. 6B). The NS2 deletion mutants, however, yielded only small pinpoint foci in MDBK monolayers, consisting of a small number of infected cells (Fig. 6B). Due to their very slow growth in MDBK, cells had to be split every three days in a 1:3 ratio to keep the infection going on. At 15 days after infection of MDBK cells at an MOI of 0.01, antigen was detected in less than 60% of cells. In contrast, in the case of standard ATue51908 or of full-length recombinant virus, it was not possible to split MDBK cells infected with an MOI of 0.01 more than once due to an extensive CPE. When BSR T7/5 cells were infected with the NS2 deletion mutants at an MOI of 0.01, CPE was maximal at 5 days after infection. In these cultures, antigen was detected in more than 80% of cells. Still, compared to full-length virus, maximum titers of the NS2 deletion mutants were 10-fold lower in both cell lines, with maximum titers reaching 10^4 PFU per ml in BSR T7/5 cells at 5 days after infection and 10^5 PFU per ml in MDBK cells at 15 days after infection and four passages of infected cells. These features demonstrate that the NS2 gene is nonessential, though in its absence the replication capacity of BRSV is reduced and the CPE in MDBK cells is less pronounced.

DISCUSSION

In this work, we describe the cDNA cloning of the entire BRSV genome and the recovery of recombinant BRSV from cDNA constructs. To our knowledge, this is the second species of the genus *Pneumovirus* for which the entire genome sequence has been determined and which has been made amenable to genetic manipulation. The viruses we have recovered are derived from a parental virus obtained after 12 passages in MDBK cells of BRSV strain A51908, obtained from the ATCC. Compared to the published sequences of strain A51908, a marked divergence was observed. The most striking deviations are located in the SH and G genes, which displayed 86 and 88% amino acid identity, respectively. The parental virus was therefore designated ATue51908 (GenBank accession no. AF092942).

The approach used to rescue cDNA derived from ATue51908 into infectious BRSV follows the principles shown to be successful for recovery first of recombinant rabies virus (40) and then of a variety of negative-strand RNA viruses from the rhabdovirus, paramyxovirus, and bunyavirus families (8). It involves simultaneous expression of viral antigenome RNA and of RNP proteins in order to promote "illegitimate" encapsidation of the RNA in such a way that the novel RNP structure may serve as a template for the viral RNA polymerase. The expression systems most widely used rely on infection of cells with recombinant vaccinia viruses (vTF7-3 or MVA) (2–4, 10, 13, 17, 18, 23, 45) providing T7 RNA polymerase, which is needed for expression of proteins and RNAs from transfected plasmids. This, however, requires coping with vaccinia virus-induced CPEs which limit the window for recovery, vaccinia virus-induced recombination of transfected plasmid DNA (13, 18), and separation of different viruses by biophysical (40), biochemical (45), or biological (13) means. We describe here the establishment of a BHK-derived cell line stably expressing T7 RNA polymerase, as first reported for measles virus (32), obviating the use of vaccinia helper virus. Especially for recovery of BRSV, the availability of a vaccinia virus-free system was regarded as crucial, since BRSV replicates very slowly and to low titers in cell culture and is highly cell associated, like vaccinia virus, and since virions are pleomorphic in size and shape, with some particles comparable in size to vaccinia virus. Moreover, a general advantage of the T7 polymerase-expressing cell line in combination with IRES-containing support plasmids (11) was confirmed in the rabies virus recovery system.

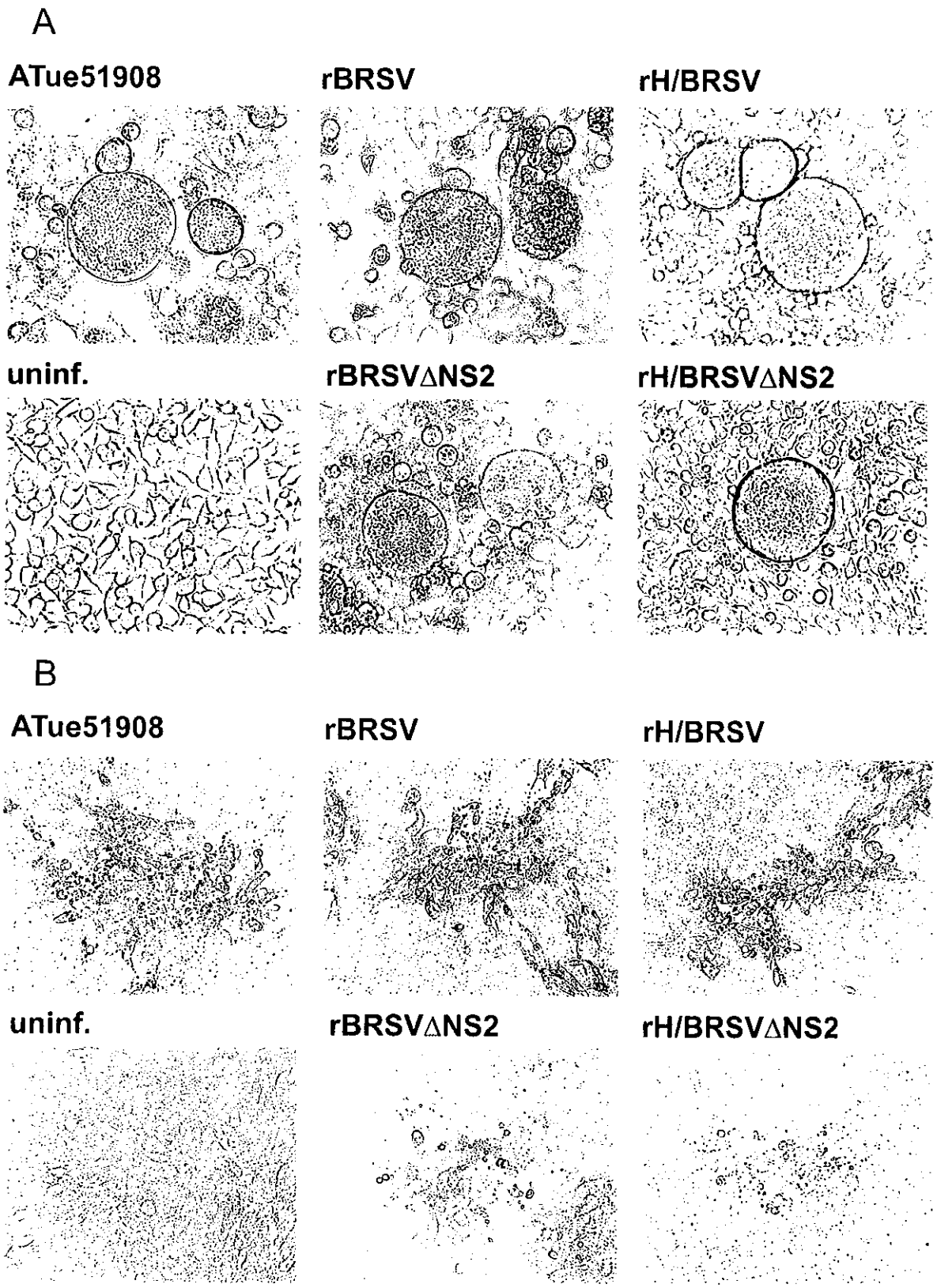

FIG. 6. CPE of recombinant BRSVs in BSR T7/5 cells (A) and MDBK cells (B). (A) In BSR T7/5 cultures, all recombinants induced syncytia of cells detaching from the monolayer, which were indistinguishable from those of standard ATue51908, at 56 h postinfection at an MOI of 0.01. (B) In MDBK cells, both full-length recombinant viruses rBRSV and rH/BRSV and standard ATue51908 virus produced similar foci of enlarged cells at 5 days postinfection, whereas the NS2 deletion mutants produced only small foci of degenerating cells.

Compared to the vaccinia virus-driven expression system, the recovery rates were increased at least 10-fold (12a).

In contrast to other paramyxoviruses or rhabdoviruses, where N, P, and L proteins are sufficient to support encapsidation of antigenome RNA and to initiate an infectious cycle, recovery of the pneumovirus HRSV has been shown to require the additional expression of the M2 gene (ORF 1), which encodes a transcription elongation factor (5, 16). We therefore included a BRSV M2-expressing plasmid in the transfection experiments. Both viruses corresponding to ATue51908, chimeric viruses possessing the leader region of HRSV, and considerably attenuated NS2-deletion mutants could be isolated from every transfected cell culture dish. The recovery rate thus exceeds by far 1 in 10^6 transfected cells.

The successful recovery of recombinant BRSV indistinguishable in phenotype from the parental virus ATue51908 confirmed the authenticity and the functionality of the determined nucleotide sequences. A particular focus was laid on the functional characterization of BRSV sequences previously not available, namely, the gene ends and the L gene. The deduced BRSV L and HRSV L proteins showed amino acid identity of 84% and a similarity of 89%. The amino acid differences were distributed over the entire protein, indicating rather identical functions of the two proteins. This finding, and the high degree of similarity of the HRSV and BRSV genome ends, prompted us to see whether the BRSV polymerase would be able to recognize HRSV promoter structures.

The 3′ end of the pneumovirus genome (the genome promoter) contains *cis*-acting signals directing both transcription of subgenomic, free mRNAs and synthesis of full-length RNPs, as well as sequences specifying an encapsidation signal. In the case of HRSV minigenomes, the promoter function was highly sensitive to insertion of nucleotides into the terminal 10 residues and into the stretch of residues 21 to 25 (6, 7). Interestingly, the eight nucleotide differences of the BRSV leader region are all located outside of these functionally critical regions. To determine whether the published HRSV sequence can substitute for the BRSV genome promoter, chimeras which contain the heterologous HRSV leader region were designed. The chimeric viruses could be rescued with the same efficiency as rBRSV and replicated with the same speed to the same titers and produced the same type of CPE. The same applied to the chimeric and authentic NS2 deletion mutants (see below). Therefore, the heterologous HRSV leader region is faithfully recognized by the BRSV polymerase and provides functionally identical signals for RNA transcription and replication and for encapsidation of RNAs. The observed nucleotide differences apparently do not specify genetic information that may account for species specificity, further confirming the close relationship of BRSV and HRSV.

Members of the genus *Pneumovirus* show particular features that distinguish them from other paramyxovirus genera, such as the lack of P gene RNA editing. HRSV does not appear to obey the "rule of six" (35), as do most paramyxoviruses which are able to edit their P gene RNAs (21). Apparently, this is also true for BRSV. Neither the parental virus ATue51908 nor any of the recombinants have a genome consisting of multiples of 6 nt. The recombinant BRSV genome lacks five residues compared to the ATue51908 genome but replicated with the same efficiency. This applies also to the chimeric H/BRSV, which lacks six residues due to the shorter HRSV leader region. This 1-nt difference was also maintained in the NS2 deletion mutants BRSVΔNS2 and H/BRSVΔNS2. Again, they were found to replicate at identical rates.

Another peculiarity of pneumoviruses is the high number of encoded genes, some of which do not have counterparts in other paramyxoviruses, such as SH, M2, and the nonstructural genes NS1 and NS2. It was recently shown that G and SH of HRSV are not essential for viral replication in vitro but may enhance membrane fusion (19). A role for the M2 protein as a transcription elongation factor has also been established (5, 16), but the function of the two nonstructural proteins NS1 and NS2 has remained rather obscure. In an artificial minigenome assay, HRSV NS1 and, to a lesser degree, NS2, showed inhibitory effects on transcription and replication (1). Only revertants of HRSV NS2 knockout viruses, in which tandem stop codons were introduced in such a way that the NS2 protein would not be expressed, could be isolated from plaques observed in transfection experiments (42), indicating an important function of HRSV NS2 in the virus life cycle. Strikingly, in the avian pneumovirus TRTV, both nonstructural genes are absent, emphasizing the question of whether they represent essential genes in mammalian pneumoviruses such as BRSV.

To address possible functions of NS2 in the virus life cycle, we assembled a BRSV cDNA copy lacking the entire NS2 gene. The successful recovery of a virus autonomously propagating in BSR cells demonstrated that the NS2 gene is not essential for replication of BRSV in vitro. The pattern and the relative amounts of mRNAs and full-length virus RNA produced in infected cells were not markedly changed, except for the lack of an NS2 transcript. Interestingly, the effect of the NS2 deficiency appeared to be more pronounced in the bovine MDBK cell line than in BSR cells. Syncytia caused by the NS2-deficient mutants in BSR T7/5 cell monolayers were phenotypically indistinguishable from those caused by standard ATue51908. In contrast, BRSVΔNS2-infected MDBK cells were not enlarged, unlike ATue51908-infected cells. Moreover, maximum titers were reached in MDBK cells infected with NS2 deletion mutants by 15 days after infection, compared to 8 days after infection with standard ATue51908. In BSR T7/5 cells, maximal final infectious titers were reached by 5 days after infection, which is comparable to nondeficient viruses. In both cell lines, however, the maximum virus yield of the NS2 deletion mutants was reduced by a factor of 10 (10^5 and 10^4 in BSR T7/5 cells for rBRSV and rBRSVΔNS2, respectively, and 10^6 and 10^5 in MDBK cells, respectively). As the cDNA constructs used for recovery of nondeficient rBRSV were made by completion of the NS2 deletion cDNA, the observed slower growth and the reduced virus titers are due to the lack of NS2 rather than to putative differences in other parts of the genome. Thus, although not essential, NS2 is an accessory factor able to substantially support virus growth, by a thus far unknown mechanism. Further experiments using modified virus mutants are now feasible and should help to reveal the mechanisms of NS2 involved in facilitating virus growth in different cell types.

The successful recovery of the first BRSV strain from which an entire gene has been deleted is important not only for studying the molecular biology and genetics of the virus but also because it provides a severely attenuated virus with an unequivocal serological marker. Due to their 3′-proximal locations, NS1 and NS2 are expressed at high levels and induce antibodies in infected calves (44). Further manipulation of NS2-deficient viruses may lead to the development of attenuated marker vaccines, easily distinguishable from wild-type virus. For the prevention of respiratory diseases, live vaccines appear to be best suited due to their ability to confer local immunity in addition to humoral immune response. It will also be of interest to determine whether foreign epitopes or proteins can be incorporated into the virion in order to design vaccines for other respiratory pathogens or vectors for transient gene therapy.

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Highly Permissive Cell Lines for Subgenomic and Genomic Hepatitis C Virus RNA Replication

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Hepatitis C virus (HCV) replication appears to be restricted to the human hepatoma cell line Huh-7, indicating that a favorable cellular environment exists within these cells. Although adaptive mutations in the HCV nonstructural proteins typically enhance the replicative capacity of subgenomic replicons in Huh-7 cells, replication can only be detected in a subpopulation of these cells. Here we show that self-replicating subgenomic RNA could be eliminated from Huh-7 clones by prolonged treatment with alpha interferon (IFN-α) and that a higher frequency of cured cells could support both subgenomic and full-length HCV replication. The increased permissiveness of one of the cured cell lines allowed us to readily detect HCV RNA and antigens early after RNA transfection, eliminating the need for selection of replication-positive cells. We also demonstrate that a single amino acid substitution in NS5A is sufficient for establishing HCV replication in a majority of cured cells and that the major phosphate acceptor site of subtype 1b NS5A is not essential for HCV replication.

An estimated 3% of the world's population is seropositive for hepatitis C virus (HCV) (32). The acute phase of infection is often subclinical; however, approximately 70% of seropositive individuals develop a chronic infection, predisposing the infected patient to the development of progressive liver pathology, including fibrosis, cirrhosis, and hepatocellular carcinoma (1, 28). The current treatments for HCV infection are alpha interferon (IFN-α) in combination with ribavirin or, more recently, a polyethylene glycol-modified form of IFN-α; however, sustained responses are only observed in ~50% of treated patients, and effectiveness varies depending on the infecting HCV genotype (19).

HCV has been classified within its own genus, *Hepacivirus*, within the family *Flaviviridae*, which comprises three genera of small enveloped positive-strand RNA viruses (27). The 9.6-kb genome consists of a single open reading frame (ORF) flanked by 5′ and 3′ nontranslated regions (NTRs) (reviewed in references 3, 4, and 16). The 5′ NTR contains an internal ribosome entry site (IRES), mediating cap-independent translation of the ORF of ~3,011 amino acids. The resulting polyprotein is processed co- and posttranslationally into at least 10 individual proteins. Host signal peptidase cleavages within the N-terminal portion of the polyprotein generate the structural proteins core (C), E1, and E2. Two HCV-encoded proteases mediate downstream cleavages, liberating the nonstructural (NS) proteins involved in viral replication. The NS2-3 protease spanning the C-terminal half of NS2 and the N-terminal one-third of NS3 catalyzes autocatalytic cleavage between NS2 and NS3. The N-terminal one-third of NS3 also encodes a serine protease that functions in concert with NS4A to cleave downstream sites, while the C-terminal two-thirds harbors RNA helicase and RNA-stimulated nucleoside triphosphatase activities. The NS5B protein exhibits an RNA-dependent RNA polymerase activity. Although the NS4B and NS5A proteins are membrane associated and form complexes with other HCV NS proteins (10), their biochemical functions remain speculative (29). NS5A is a serine phosphoprotein associated with one or more cellular kinases (23), and at least two distinct phosphorylated forms, p56 (basal phosphorylated form) and p58 (hyperphosphorylated form), have been described (11, 30). For the consensus HCV-H genotype 1a NS5A, Ser-2321 was identified as the major phosphate acceptor site (24), whereas the preferred site for p56 phosphorylation in the genotype 1b HCV-BK isolate was identified as Ser-2194 (12). For the hyperphosphorylated form of NS5A, deletion mapping and site-directed mutagenesis identified Ser-2197, Ser-2201, and Ser-2204 as putative phosphorylation sites (30). We recently demonstrated that Ser-2204 and apparent hyperphosphorylation were not essential for HCV replication in vitro (5); however, the importance and role of NS5A phosphorylation in replication are unknown.

Recently, Lohmann and colleagues reported that bicistronic subgenomic HCV replicons, containing the neomycin phosphotransferase gene (*neo*) in lieu of the HCV structural genes, were capable of autonomous replication in ~1 in 10^6 Huh-7 cells (18). This development facilitated the identification of adaptive mutations in the HCV NS proteins that increased RNA replication as well as the frequency of Huh-7 cells supporting detectable levels of replication (5, 9, 15, 17). Previously, we identified a series of amino acid substitutions and a deletion of 47 amino acids in NS5A that enhanced the initiation of productive replication and G418-resistant colony formation (5). Replacement of the Ser residue with Ile at position 2204 in NS5A permitted HCV RNA replication in ~10% of transfected Huh-7 cells (a 20,000-fold improvement) and in-

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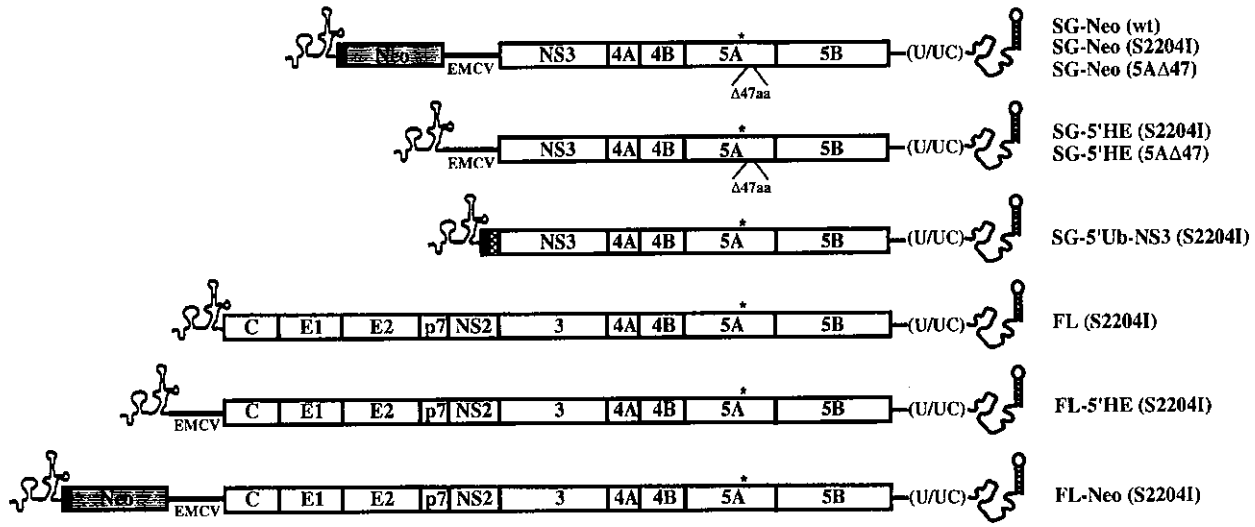

FIG. 1. Schematic representation of HCV RNAs used in this study. The 5′ and 3′ NTR structures are shown, and ORFs are depicted as open boxes with the polyprotein cleavage products indicated. The first 12 amino acids of the core-coding region (solid box), the *neo* gene (Neo; shaded box), the EMCV IRES (EMCV; solid line), and ubiquitin (cross-hatched box) are illustrated. Locations of the NS5A adaptive mutations S2204I (*) and Δ47aa are indicated.

creased replication to a level sufficient for the detection of HCV RNA early after transfection (5). Although the development of adapted subgenomic replicons provided a system to study HCV replication, we were only able to detect HCV replication in ~10% of transfected Huh-7 cells, suggesting that the cellular environment was a major determinant of HCV replication efficiency.

In order to obtain cell lines more permissive for HCV replication, clonal and population Huh-7 cell lines supporting adapted and nonadapted subgenomic RNA replication were cured of HCV RNA by treatment with IFN-α. In general, a higher percentage of cured cells were able to support HCV replication and facilitated the transient detection of both subgenomic and full-length replication by multiple assays. We also examined the adaptive value of different amino acid substitutions at the NS5A 2204 locus and the effect of combining different adaptive mutations. Furthermore, we demonstrate that phosphorylation of the major phosphate acceptor site in subtype 1b NS5A is not an absolute requirement for replication in vitro. Our findings highlight the importance of the host environment for the establishment of HCV replication and provide a valuable cell line for studying early events in HCV replication.

MATERIALS AND METHODS

Cell culture and IFN treatment. Huh-7 cell monolayers were propagated in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 0.1 mM nonessential amino acids (DMEM–10% FBS). For cells supporting subgenomic replicons, G418 (Geneticin; Gibco-BRL) at 750 μg/ml was added to the culture medium. Replicon-containing Huh-7 cells were cured of HCV RNA by initially passaging cells twice in the absence of G418. On the third passage cells were cultured with human leukocyte-derived IFN-α (100 IU/ml; Sigma-Aldrich). After 3 to 4 days, confluent monolayers were trypsinized, plated and cultured for 24 h before the addition of IFN-α. Cells were passaged a total of four times in the presence of IFN-α, and prior to the fourth passage cells were grown for 3 days without IFN-α. Cured cell lines were expanded and cryopreserved at early passage levels. Experiments were conducted using cells that been passaged fewer than 20 to 30 times from these cryopreserved seed lots.

Plasmid constructions. Standard recombinant DNA technology was used to construct and purify all plasmids. Primed DNA synthesis was performed with KlenTaqLA DNA polymerase (kindly provided by Wayne Barnes, Washington University, St. Louis, Mo.), and regions amplified by PCR were confirmed by automated nucleotide sequencing. Plasmid DNAs for in vitro transcription were prepared from large-scale bacterial cultures and purified by centrifugation in CsCl gradients.

All nucleotide and amino acid numbers refer to the location within the genotype 1b Con1 full-length HCV genome (GenBank accession no. AJ238799) commencing with the core-coding region. This sequence was assembled from chemically synthesized DNA oligonucleotides in a stepwise PCR assay essentially as described previously (5). Briefly, 10 to 12 gel-purified oligonucleotides (60 to 80 nucleotides [nt] long) with unique complementary overlaps of 16 nt were used to synthesize cDNAs spanning 600 to 750 bases. The final PCR products were purified, digested with appropriate restriction enzymes, and ligated into the similarly cleaved pGEM3Zf(+) plasmid vector (Promega). Multiple recombinant clones were sequenced, correct clones were identified, and overlapping cDNA fragments were assembled into the following contiguous genomic sequence: 5′ NTR-C-E1-E2-p7-NS2-3-4A-4B-5A-5B-3′ NTR (pHCVBMFL). The selectable replicon pHCVrep1bBartMan/*Ava*II (SG-Neo [wild type, [wt]) (Fig. 1) and the derivatives pHCVrep1b/BBVII [SG-Neo (S2204I)] and pHCVrep1b/BBI [SG-Neo (5AΔ47)] containing the NS5A adaptive mutations, S2204I, and an in-frame deletion of 47 amino acids (Δ47aa) between nt 6960 and 7102, respectively have been described previously (5) (Fig. 1). The plasmid pHCVBMFL/S2204I [FL (S2204I)] (Fig. 1) contains the full-length genome with the NS5A adaptive change S2204I. For the genomic and subgenomic constructs, NS5B polymerase-defective derivatives carrying a triple amino acid substitution were generated, changing the Gly-Asp-Asp (GDD) motif in the active site to Ala-Ala-Gly (AAG) (5), and throughout this report these constructs are referred to as pol$^-$.

The plasmid pC-Ubi-NS3/HCVrepBBVII [SG-5′Ub-NS3 (S2204I)] (Fig. 1) containing ubiquitin instead of the *neo* gene and encephalomyocarditis virus (EMCV) IRES was constructed as follows. An *Asc*I-*Sac*I-digested PCR fragment amplified from pHCVrep12/Neo (K. J. Blight et al., unpublished results) with primers 1289 and 1290 (Table 1) and the *Sac*I-*Bsr*GI portion of a second PCR product generated using the primer pair 1291-1292 (Table 1) with pHCVrep1b/BBVII were ligated between the *Xba*I and *Bsr*GI sites of HCVrep1b/BBVII together with the *Xba*I-*Asc*I fragment from HCVrep1b/BBVII. To delete the *neo* gene from pHCVrep1b/BBVII, synthetic overlapping oligonucleotides 1287 and 1288 (Table 1) were hybridized and extended to create the junction between the 5′ NTR and the EMCV IRES. This product was digested with *Apa*LI and *Acl*I

and inserted, together with *Xba*I-*Apa*LI and *Acl*I-*Eco*RI fragments from pHCVrep1b/BBVII, into *Xba*I-*Eco*RI digested pHCVrep1b/BBVII. This construct was named p5′NTR-EMCV/HCVrepBBVII [SG-5′HE (S2204I)] (Fig. 1). To replace S2204I with NS5AΔ47, the *Eco*RI-*Xho*I fragment from pHCVrep1b/BBI was ligated into similarly cleaved p5′NTR-EMCV/HCVrepBBVII, generating p5′NTR-EMCV/HCVrepBBI [SG-5′HE (5AΔ47)] (Fig. 1).

The plasmid p5′NTR-EMCV/HCVFLBM(S2204I) [FL-5′HE (S2204I)] (Fig. 1) was created by ligating the *Xba*I-*Hin*dIII fragment from p5′NTR-EMCV/HCVrepBBVII, the *Hin*dIII-*Aat*II fragment of a PCR product amplified from p5′NTR-EMCV/HCVrepBBVII using primers 1293 and 1294, and the *Aat*II-*Not*I fragment from pHCVBMFL/S2204I into pHCVBMFL/S2204I previously digested with *Xba*I and *Not*I. The selectable bicistronic full-length HCV clone pHCVBMFL(S2204I)/Neo [FL-Neo (S2204I)] (Fig. 1) was assembled by ligating the *Xba*I-*Hin*dIII fragment from pHCVrep1b/BBVII and the *Hin*dIII-*Eco*RI fragment from p5′NTR-EMCV/HCVBMFL(S2204I) between the *Xba*I and *Eco*RI sites of pHCVrep1b/BBVII.

To obtain plasmids with mutations at position 2204, and to introduce single A2199T or double S2197P + A2199T mutations into p5′NTR-EMCV/HCVrepBBVII, PCRs were first performed using p5′NTR-EMCV/HCVrepBBVII as a template with the reverse primer 1030 and one of the following mutant forward primers: 1319 (S2204V), 1320 (S2204A), 1322 (S2204Y), 1324 (S2204E), 1325 (S2204T), 1184 (S2204D), 1326 (A2199T + S2204I), or 1327 (S2197P + A2199T S2204I) (Table 1). PCR-amplified products were digested with *Blp*I and *Xho*I and cloned into these sites in p5′NTR-EMCV/HCVrepBBVII. S2204 was engineered by insertion of the *Eco*RI-*Xho*I fragment from pHCVrep1bBartMan/*Ava*II into similarly cleaved p5′NTR-EMCV/HCVrepBBVII.

To engineer the mutation Q1112R into p5′NTR-EMCV/HCVrepBBVII in order to create p5′NTR-EMCV/HCVrepCloneA (Q1112R + S2204I), nt 3640 to 3991 of NS3 were PCR amplified from p5′NTR-EMCV/HCVrepBBVII using mutant primer 1358 and oligonucleotide 885 (Table 1). The resulting product was digested with *Bsr*GI and *Eag*I and combined in a ligation reaction mixture with the *Eag*I-*Eco*RI and *Bsr*GI-*Eco*RI fragments from p5′NTR-EMCV/HCVrepBBVII. The double mutation (E1202G + T1280I) in NS3 was created via a multistep cloning procedure. First, a PCR fragment amplified from p5′NTR-EMCV/HCVrepBBVII with forward primer 1359 and reverse primer 1356 (Table 1) was digested with *Apa*LI and *Xba*I and cloned into *Eco*RI-*Xba*I-digested pGEM3Zf(+) together with the *Eco*RI-*Apa*LI fragment from pGEM3Zf(+)/HCV1bnt1796-2524, which contains nt 3420 to 4124 in NS3 (K. J. Blight and C. M. Rice, unpublished results), generating the intermediate plasmid pGEM3Zf(+)/HCV1bnt1796-2524NS3*. Second, in a four-part cloning strategy, the *Bsr*GI-*Bsa*AI fragment, excised from pGEM3Zf(+)/HCV1bnt1796-2524NS3*, was inserted, together with fragments *Bsa*AI-*Bss*HII and *Bss*HII-*Eco*RI from p5′NTR-EMCV/HCVrepBBVII, into p5′NTR-EMCV/HCVrepBBVII cleaved with *Bsr*GI and *Eco*RI. The resultant plasmid was named p5′NTR-EMCV/HCVrepBBVII+NS3* (E1202G + T1280I + S2204I).

The mutations S2194A and S2194D were introduced by using primer pairs 5′Ala-1030 and 5′Asp-1030 (Table 1), respectively, to PCR amplify nt 6897 to 7186 in NS5A from pHCVrep1b/BBVII. These mutations were incorporated into pHCVrep1b/BBVII by replacing the *Blp*I-*Xho*I portion with the corresponding *Blp*I-*Xho*I-digested PCR product.

RNA transcription. Plasmid DNAs containing full-length and subgenomic HCV sequences were linearized with *Sca*I and a poliovirus subgenomic replicon digested with *Bam*HI. The linearized DNAs were phenol-chloroform (1:1) extracted and precipitated with ethanol. Pelleted DNAs were washed in 80% ethanol and resuspended in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA (pH 8.0). RNA transcripts were synthesized at 37°C for 90 min in a 100-μl reaction mixture containing 40 mM Tris-HCl (pH 7.9), 10 mM NaCl, 12 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol (DTT), a 3 mM concentration of each nucleoside triphosphate, 0.025 U of inorganic pyrophosphatase (Roche Applied Science), 100 U of RNasin (Promega), 100 U of T7 RNA polymerase (Epicentre Technologies), and 2 μg of linearized DNA. RNA was extracted with phenol-chloroform (1:1) and ethanol precipitated, and the pellet was washed in 80% ethanol before resuspension in double-distilled H_2O. DNA template was removed by three serial DNase digestions for 20 min at 37°C in a solution of 33 mM Tris-HCl (pH 7.8), 66 mM KCl, 10 mM $MgCl_2$, and 5 mM dithiothreitol containing 10 U of DNase I (Roche Applied Science). DNase-digested RNAs were extracted with phenol-chloroform (1:1) and ethanol precipitated, and the RNA pellet was resuspended in double-distilled H_2O after washing in 80% ethanol. The RNA concentration was determined by measurement of the optical density at 260 nm, and the integrity and concentration were confirmed by 1% agarose gel electrophoresis and ethidium bromide staining.

Transfection of cultured cells. In vitro-transcribed RNA was transfected into Huh-7 and IFN-α-cured cells by electroporation. Briefly, subconfluent Huh-7

TABLE 1. Oligodeoxynucleotides used in this study[a]

Name	Sequence
885	(−)CCCTCTAGAACGCCCCGAAACCTAGGGTGCG
1030	(−)CCCTCTAGACTCGAGGGGAATTTCCTGGAC
1184	(+)GACGGCTAAGCGTAGGCTGGCCAGGGGATCTCCCCCCTCCTTGGCCAGCTCATCAGCTGACCAGCTGTCTGCGCCTTCC
1287	(+)AGACCGTTGCACCAGACCACAACGGTTTCCCTCTAGCGGGATCAATTCCG
1288	(−)CCAGTAACGTTAGGGGGGGGAGGGAGGCGGAATTGATCCCGCT
1289	(+)CCAAAGGGGCGCCCATGCAGATCTTCGTGAAGACC
1290	(−)AATAGGAGCTCCACCGCGGAGACGC
1291	(+)CGGTGGAGCTCCTATTACGGCCTACTCCCAAC
1292	(−)ATTGGTGTACATTTGGGTGATTGG
1293	(+)TCTGGAAGCTTCTTGAAGACA
1294	(−)GCTTGACGTCCTGTGGGCGGGTTGGTGTTACGTTTGGTTTTTCTTTGAGGTTTAGGATTCGTGCTCATTATTATATCGTGTTTTTTCAAAGG
1319	(+)AGACGGCTAAGCGTAGGCTGGCCAGGGGATCTCCCCCCTCCTTGGCCAGCTCATCAGCTGTACAGCTGTCTGCGCCTTCC
1320	(+)AGACGGCTAAGCGTAGGCTGGCCAGGGGATCTCCCCCCTCCTTGGCCAGCTCATCAGCTTACCAGCTGTCTGCGCCTTCC
1322	(+)AGACGGCTAAGCGTAGGCTGGCCAGGGGATCTCCCCCCTCCTTGGCCAGCTCATCAGCTTGCCAGCTGTCTGCGCCTTCC
1324	(+)AGACGGCTAAGCGTAGGCTGGCCAGGGGATCTCCCCCCTCCTTGGCCAGCTCATCAGCTGAACAGCTGTCTGCGCCTTCC
1325	(+)AGACGGCTAAGCGTAGGCTGGCCAGGGGATCTCCCCCCTCCTTGGCCAGCTCATCAGCTACACAGCTGTCTGCGCCTTCC
1326	(+)AGACGGCTAAGCGTAGGCTGGCCAGGGGATCTCCCCCCTCCTTGGCCAGCTCATCAGCTATCCAGCTGTCTGCGCCTTCC
1327	(+)AGACGGCTAAGCGTAGGCTGGCCAGGGGATCTCCCCCCTCCTTGACCAGCTCATCAGCTATCCAGCTGTCTGCGCCTTCC
1356	(−)CCGCTCTAGATACGTGATGGGGGCACCCGTGGTGATGGTCCTTACCCCGATTCTGATGTTAGGGTCGATAC
1358	(+)CCGATGTACACCAGGACCTCGTCGGCTGGCGAGCGCCCCCGGGCGCGTTC
1359	(+)CCGCGTGCACCGAGGGGTTGCGAAGGCGGTGGACTTTGTACCCGTCGAGTCTATGGGAACCACTATGCGGTCCCCGGTC
5′Ala	(+)CCACGCTAAGCGTAGGCTGGCCAGGGGAGATCCCCCCCTCCTTGGCCAGCTC
5′Asp	(+)CCACGCTAAGCGTAGGCTGGCCAGGGGAGATCCCCCCCTCCTTGGCCAGCTC

[a] Nucleotide changes are highlighted in boldface type, and the resultant codon is underlined. Restriction sites used for cDNA cloning are underlined. The polarities of oligonucleotides are indicated as either the HCV genome RNA sense (+) or its complement (−).

cells were detached by trypsin treatment, collected by centrifugation (500 × *g*, 5 min), washed three times in ice-cold RNase-free phosphate-buffered saline (PBS), and resuspended at 1.25×10^7 cells/ml in PBS. RNA transcripts (1 μg) were mixed with 0.4 ml of washed Huh-7 cells in a 2-mm gap cuvette (BTX) and immediately pulsed (0.92 kV; pulse-length, 99 μs; five pulses) using a BTX ElectroSquarePorator. Pulsed cells were left to recover for 10 min at room temperature and then diluted into 10 ml DMEM-10% FBS. Cells were plated in (i) 35-mm-diameter wells for quantifying HCV RNA and for metabolic labeling experiments, (ii) eight-well chamber slides (Becton Dickinson) for immunofluorescence studies, or (iii) 100-mm-diameter dishes for fluorescence-activated cell sorting (FACS) analysis and G418 selection. To determine the efficiency of G418-resistant colony formation, transfected cells were plated at multiple densities (between 1×10^3 and 2×10^5 cells), together with cells transfected with pol$^-$ RNA transcripts such that the total cell number was maintained at 2×10^5 cells per 100-mm-diameter dish. Forty-eight hours after plating, medium was replaced with DMEM–10% FBS supplemented with G418 at 1 mg/ml. Three weeks later, G418-resistant foci were fixed with 7% formaldehyde and stained with 1% crystal violet in 50% ethanol to facilitate colony counting. The G418 transduction efficiency was calculated based on the number of G418-selected colonies relative to the number of Huh-7 cells plated after electroporation.

Transfection efficiency was monitored for each series of RNAs by electroporating in parallel a poliovirus subgenomic replicon expressing green fluorescent protein (GFP) (A. A. Kolykhalov and C. M. Rice, unpublished results). Transfected cells were observed for poliovirus replicon-induced cytopathic effect, and GFP expression was visualized using a fluorescent inverted microscope at 12 to 16 h posttransfection. After 24 h, the surviving attached cells (presumably not transfected with the poliovirus replicon) were trypsinized and mixed with trypan blue and viable cells were counted to determine the percentage of cells electroporated.

Viral RNA analysis. Total cellular RNA was isolated using TRIZOL reagent (Gibco-BRL) according to the manufacturer's protocol. One-tenth of each RNA sample was used to quantify HCV-specific RNA levels using an ABI PRISM 7700 sequence detector (Applied Biosystems). Real-time reverse transcription (RT)-PCR amplifications were performed using the TaqMan EZ RT-PCR core reagents (Applied Biosystems) and primers specific for the HCV 5′ NTR: 5′-CCTCTAGAGCCATAGTGGTCT-3′ (sense, 50 μM), 5′CCAAATCTCCAGGCATTGAGC-3′ (antisense, 50 μM), and FAM-CACCGGAATTGCCAGGACGACCGG (probe, 10 μM; Applied Biosystems). RT reactions were incubated for 30 min at 60°C, followed by inactivation of the reverse transcriptase coupled with activation of *Taq* polymerase for 7 min at 95°C. Forty cycles of PCR were performed with cycling conditions of 15 s at 95°C and 1 min at 60°C. Synthetic HCV RNA standards of known concentration were included with each set of reactions and used to calculate a standard curve. The real time PCR signals were analyzed using SDS software (version 1.6.3; Applied Biosystems).

FACS analysis. Transfected cell monolayers were removed from 100-mm-diameter culture dishes by Versene-EDTA treatment and a single-cell suspension prepared by passing cells through a 16-gauge needle and a 74-μm-pore-size membrane. Cells were resuspended at 2×10^6 per ml, and an equal volume of 4% paraformaldehyde added to the cell suspensions and incubated for 20 min at room temperature. Fixed cells were washed twice with PBS, and the resultant cell pellet was resuspended at 2×10^6 cells per ml in 0.1% saponin–PBS. After incubation for 20 min at room temperature, cells were stained (1 h at room temperature) with HCV-specific monoclonal antibodies (MAbs) (core [C750], NS3 [1B6], and NS5B [12B7] [all generously provided by Darius Moradpour, University of Freiburg, Freiburg, Germany]) diluted to 10 μg/ml in 3% FBS–0.1% saponin–PBS. Cells were washed three times with 0.1% saponin–PBS, and bound MAb was detected by incubation for 1 h at room temperature with anti-mouse immunoglobulin G (IgG) conjugated to Alexa 488 (Molecular Probes) diluted 1:1,000 in 3% FBS–0.1% saponin–PBS. Stained cells were washed three times with 0.1% saponin–PBS, resuspended in FACSflow buffer (BD Biosciences), and analyzed immediately using a FACS Calibur apparatus (BD Biosciences).

Indirect immunofluorescence. Electroporated Huh-7.5 cells seeded in eight-well chamber slides were washed with PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. Cells were washed twice with PBS, permeabilized by incubation with 0.1% saponin–PBS for 20 min at room temperature, and blocked with 3% goat serum for 20 min at room temperature. The NS5B MAb (12B7) was diluted to 10 μg/ml in 0.1% saponin–3% goat serum–PBS and incubated for 1 h at room temperature, followed by three washes with 0.1% saponin–PBS. Bound MAbs were detected by incubating for 1 h at room temperature with anti-mouse IgG conjugated to Alexa 488 diluted 1:1,000 in 0.1% saponin–3% goat serum–PBS. Nuclei were stained for 20 min at room temperature with Hoechst 33342 (10 μg/ml; Sigma-Aldrich) in PBS. Unbound fluorescent conjugate was removed by three washes with 0.1% saponin–PBS, and cells were mounted in Vectashield (Vector Laboratories) and viewed with a fluorescence microscope (Eclipse TE300; Nikon).

Metabolic labeling of proteins and immunoprecipitation. Cell monolayers in 35-mm-diameter wells were incubated for 0.5 to 10 h in methionine- and cysteine-deficient minimal essential medium containing 1/40 the normal concentration of methionine, 5% dialyzed FBS, and Express ^{35}S-protein labeling mix (140 μCi/ml; NEN). Labeled cells were washed once with cold PBS and harvested in 200 μl of sodium dodecyl sulfate (SDS) lysis buffer (0.1 M sodium phosphate buffer [pH 7.0], 1% SDS, 1× complete protease inhibitor cocktail [Roche Applied Science], 80 μg of phenylmethylsulfonyl fluoride [PMSF] per ml), and cellular DNA was sheared by repeated passage through a 27-gauge needle. Equal amounts of protein lysates (50 μl) were heated at 75°C for 10 min and clarified by centrifugation prior to mixing with 200 μl of TNA (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.67% bovine serum albumin, 1 mM EDTA, 0.33% Triton X-100, 80 μg of PMSF per ml). One-μl of HCV-positive serum (8) was added, and immune complexes allowed to form by incubation overnight at 4°C with rocking. Immune complexes were collected by adding 50 μl of prewashed Pansorbin cells (Calbiochem) and incubating for 1 to 2 h at 4°C with rocking. Immunoprecipitates were collected by centrifugation and washed three times in TNAS (TNA containing 0.125% SDS) and once with TNE (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 80 μg of PMSF per ml), solubilized by heating at 80°C for 20 min in protein sample buffer, and separated on an SDS–10% polyacrylamide gel. Metabolically labeled proteins were visualized by fluorography.

RESULTS

Cell lines highly permissive for HCV replication. To date, productive HCV subgenomic RNA replication is restricted to the Huh-7 human hepatoma cell line, indicating that a favorable cellular environment exists within these cells. Although adaptive mutations in the HCV NS proteins are required to establish HCV replication in a higher frequency of transfected Huh-7 cells (5, 9, 15, 17), we identified several Huh-7 clones harboring subgenomic replicons with no consensus amino acid changes in the HCV polyprotein, as determined by sequencing the population of RT-PCR amplified HCV RNAs (5). We reasoned that these cells might provide a more permissive environment for HCV replication and a system to study HCV replication in the absence of adaptive changes.

Since HCV replication can be readily blocked by IFN-α (5, 6, 9), several Huh-7 lines harboring subgenomic HCV replicons were cured of HCV RNA by prolonged treatment with IFN-α (see Materials and Methods). From 22 G418-resistant clones (5), we cured clones Huh-7.5 and Huh-7.8, harboring SG-Neo subgenomic replicons with no amino acid changes within the HCV NS region, as well as clone Huh-7.4, containing a replicon with the Ser-to-Ile change at position 2204 in NS5A. Uncloned population lines Huh-7/S2204I and Huh-7/5AΔ47 (5), selected with G418 after transfection of subgenomic replicons containing either S2204I in NS5A [SG-Neo (S2204I)] (Fig. 1) or the 47-amino-acid NS5A deletion [SG-Neo (5AΔ47)] (Fig. 1), were also treated with IFN-α. To exclude the possibility that IFN-α treatment alone may alter the ability of Huh-7 cells to support HCV replication, the parental Huh-7 cells were treated with IFN-α in parallel. Following IFN-α treatment, cells were shown to lack HCV RNA by a nested RT-PCR specific for the 3′ NTR in which the detection limit was ~10 molecules of HCV RNA (14) and by sensitivity to G418.

To examine the ability of IFN-α-cured cell lines to support HCV replication, three G418-selectable replicons, SG-Neo (S2204I), SG-Neo (5AΔ47), and SG-Neo (wt) (Fig. 1), with G418 transduction efficiencies in parental Huh-7 cells of 10,

0.2, and 0.0005%, respectively, were used (5). In vitro-synthesized RNA was electroporated into IFN-α-cured cells; after 48 h, G418 selection was imposed, and the resulting colonies were counted after fixing and staining. The transduction efficiencies were calculated on the basis of the number of G418-selected colonies relative to the number of Huh-7 cells plated after electroporation. The frequency of Huh-7.5 cells able to support SG-Neo (S2204I) replication was approximately threefold higher than that of the parental Huh-7 cells (Fig. 2). For cell lines Huh-7.5 and Huh-7.8, the number of G418-resistant colonies obtained after transfection of SG-Neo (5AΔ47) was significantly higher than that for the parental Huh-7 cells (~33- and 9-fold increases, respectively; Fig. 2). The same was true for Huh-7.4, although the increased frequency of colony formation was not as great (~3-fold; Fig. 2). The SG-Neo (wt) replicon also showed an enhanced replicative capacity in Huh-7.5, Huh-7.8, and Huh-7.4 cells (10-, 2-, and 2-fold increases, respectively; Fig. 2).

The two cured cell populations, Huh-7/5AΔ47 and Huh-7/S2204I, showed either comparable or modest increases in G418 transduction efficiencies after transfection of the adapted replicon RNA originally present within the population line (Fig. 2). The frequency of G418-resistant colonies increased ~2.5-fold when Huh-7/5AΔ47 cells were electroporated with SG-Neo (5AΔ47), whereas transfection with SG-Neo (S2204I) resulted in a slight decrease in the G418 transduction efficiency (Fig. 2). However, a 23-fold reduction in colony formation was observed after transfection of Huh-7/S2204I cells with SG-Neo (5AΔ47) (Fig. 2). No significant differences in G418-resistant colony formation were noted between the parental Huh-7 cells and IFN-α-treated Huh-7 cells (data not shown), indicating that the IFN-α-mediated curing protocol did not stably influence the ability of these cells to support HCV replication. G418-resistant colonies were not observed when the polymerase-defective replicon RNA, pol$^-$, was transfected in parallel (data not shown). Hence, a higher frequency of cells in the cured clonal lines, in particular those originally able to support replication of RNAs without adaptive mutations (Huh-7.5 and Huh-7.8), are permissive for HCV replication.

HCV replication in unselected Huh-7.5 and Huh-7 cells. Since the cured Huh-7.5 line was the most permissive of those tested, we examined HCV replication in this subline compared with the parental Huh-7 cells using a number of different methods. We focused on transient assays that would allow an assessment of HCV replication early after transfection without the need for G418 selection. Ninety-six hours after transfection with SG-Neo (S2204I) and SG-Neo (5AΔ47) RNA (Fig. 1), total RNA was extracted from Huh-7.5 and IFN-α-treated Huh-7 cells, and the HCV RNA levels were quantified by RT-PCR. The replication-defective replicon, pol$^-$, was transfected in parallel to allow discrimination between input RNA and RNA generated by productive replication. As shown in Fig. 3, the levels of HCV RNA relative to the pol$^-$ control were consistently higher in the transfected Huh-7.5 cells. Transfection with SG-Neo (S2204I) and SG-Neo (5AΔ47) RNAs resulted in 410- and 28-fold increases, respectively, in Huh-7.5 cells (Fig. 3, lanes 4 and 5), compared to only 85- and 6-fold increases in Huh-7 cells (Fig. 3, lanes 10 and 11). Since these increases are measured relative to the replication-defective pol$^-$ control, they reflect accumulation of newly synthesized RNA versus degradation of input RNA. In Huh-7.5 and Huh-7 cells, the level of residual pol$^-$ RNA declined by about 10-fold at each time point. In Huh-7.5 cells, RNAs with good replicative abilities [like SG-Neo (S2204I)] tended to accumulate over time such that a 10-fold increase was observed by 96 h. Those with lower replicative ability [like SG-Neo (5AΔ47)] remained constant or declined slightly, but never to the level of the pol$^-$ control. For Huh-7 cells, the picture was somewhat different. For example, SG-Neo (S2204I) RNA remained relatively constant, whereas SG-Neo (5AΔ47) RNA decreased over time, but again, not to the extent of the pol$^-$ control.

This finding was mirrored by the frequency of NS3-positive cells measured by FACS analysis. The percentage of NS3-positive cells was consistently higher in Huh-7.5 cells [21% for SG-Neo (S2204I) and 5% for SG-Neo (5AΔ47)] (Fig. 3, lanes 4 and 5) compared to Huh-7 cells [3% for SG-Neo (S2204I) and undetectable for SG-Neo (5AΔ47) and pol$^-$ RNAs] (Fig. 3, lanes 7, 10, and 11). These results confirm our earlier conclusion that a larger fraction of Huh-7.5 cells support detectable levels of HCV replication. The lower frequency of HCV antigen-positive cells quantified by FACS compared to the G418 transduction efficiency is attributable to the sensitivity of FACS analysis, which varies with different HCV-specific antibodies (unpublished observations).

We also examined HCV protein accumulation by metabolically labeling cells 96 h after transfection. Cell monolayers were labeled with ^{35}S-labeled methionine and cysteine for 10 h, followed by SDS-mediated lysis and immunoprecipitation of HCV proteins with an HCV-positive patient serum recognizing NS3, NS4B, and NS5A (8). After separation of labeled proteins by SDS-polyacrylamide gel electrophoresis (PAGE), NS3, NS4B, and NS5A were only visible in Huh-7.5 cells transfected with SG-Neo (S2204I) (Fig. 3, lane 4). HCV proteins were never detected in Huh-7.5 and Huh-7 cells transfected with SG-Neo (5AΔ47), pol$^-$, or SG-Neo (S2204I) RNA-electroporated Huh-7 cells (Fig. 3, lanes 1, 5, 7, 10, and 11). Similar results were obtained after metabolic labeling of HCV RNA in the presence of actinomycin D (data not shown). Taken together, these analyses demonstrate the advantages of using Huh-7.5 cells for rapid analysis of HCV replication by RNA accumulation, FACS analysis, and metabolic labeling of viral proteins.

Replicative efficiencies of subgenomic and genomic HCV RNAs. The ability to monitor HCV replication without selection eliminated the need for bicistronic replicons and allowed constructs with minimal heterologous elements to be tested. We engineered a subgenomic replicon in which the HCV 5′ NTR and 12 amino acids of core were fused to ubiquitin followed by the NS3-5B coding region (including the S2204I adaptive mutation in NS5A) and the 3′ NTR [SG-5′Ub-NS3 (S2204I)] (Fig. 1). In this polyprotein, cellular ubiquitin carboxyl-terminal hydrolase cleaves at the ubiquitin/NS3 junction to produce NS3 with an authentic N-terminal Ala residue (2, 21). In vitro-synthesized RNA was electroporated into Huh-7.5 and Huh-7 cells, and the level of HCV RNA was quantified 96 h later by RT-PCR. To our surprise, HCV RNA levels did not differ from the pol$^-$ control (data not shown), indicating that SG-5′Ub-NS3 (S2204I) RNA failed to replicate. It is possible that ubiquitin may interfere with the production of a

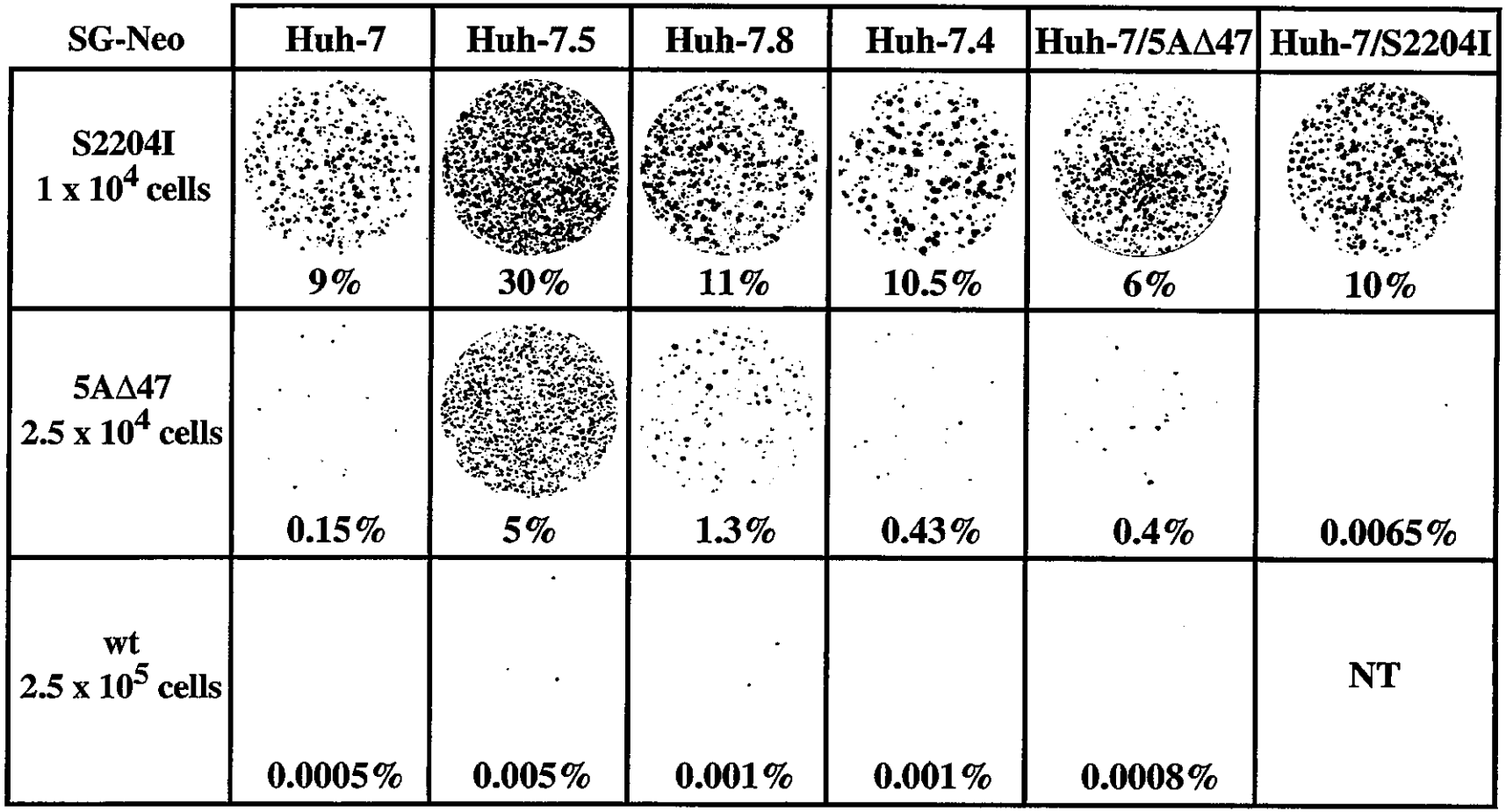

FIG. 2. Identification of Huh-7 lines highly permissive for HCV replication. Huh-7 cells that had been cured of self-replicating subgenomic RNAs by extended IFN-α treatment were electroporated with 1 μg of the subgenomic replicons SG-Neo (S2204I), SG-Neo (5AΔ47), and SG-Neo (wt). Forty-eight hours later, cells were subjected to G418 selection, and the resulting colonies were fixed and stained with crystal violet. Representative plates are illustrated, with the number of transfected cells seeded per 100-mm-diameter dish shown on the left. Percentages below each dish refer to the calculated G418 transduction efficiency of the replicon. To determine the G418 transduction efficiency, transfected cells were serially titrated from 5×10^5 to 10^3 cells per 100-mm-diameter dish, together with feeder cells electroporated with the pol$^-$ replicon. The resulting G418-resistant foci were counted for at least three cell densities, and the relative G418 transduction efficiency was expressed as a percentage, after dividing the number of colonies by the number of electroporated cells initially plated. Similar transduction efficiencies were obtained in two independent transfections. A poliovirus subgenomic replicon expressing GFP (see Materials and Methods) was electroporated in parallel. Based on both the fraction of GFP-positive cells and replicon-induced cytopathogenicity, ~90% of cells were routinely transfected. NT, not tested

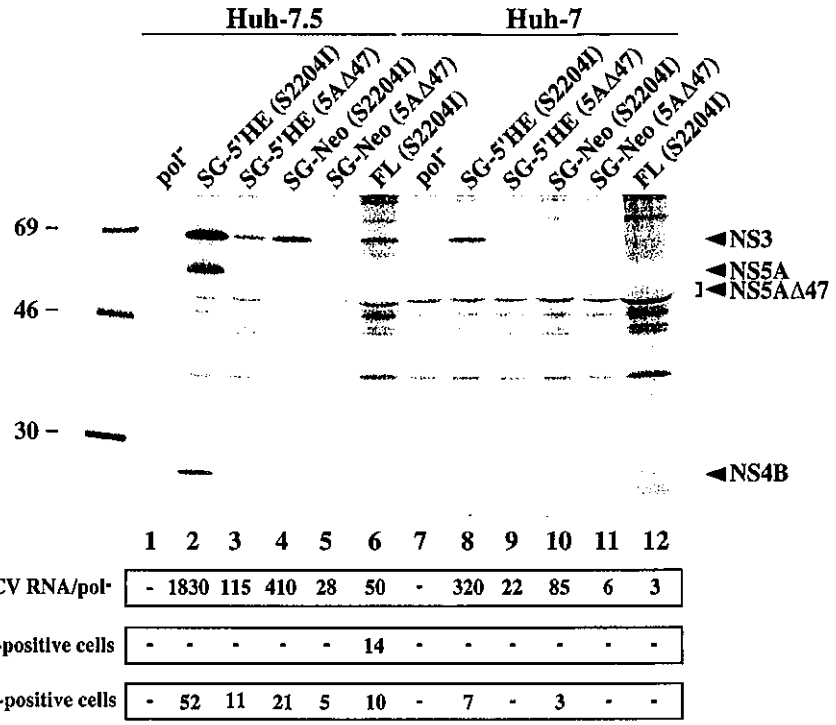

FIG. 3. Detection of HCV proteins and RNA in Huh-7.5 and Huh-7 cells transiently transfected with HCV RNA. Top panel, Huh-7.5 and Huh-7 cells were transfected with the subgenomic replicons pol⁻ (lanes 1 and 7), SG-5'HE (S2204I) (lanes 2 and 8), SG-5'HE (5AΔ47) (lanes 3 and 9), SG-Neo (S2204I) (lanes 4 and 10), SG-Neo (5AΔ47) (lanes 5 and 11), and FL (S2204I) HCV RNA (lanes 6 and 12). At 96 h posttransfection, monolayers were incubated for 10 h in the presence of [^{35}S]methionine and [^{35}S]cysteine. Labeled cells were lysed and immunoprecipitated with HCV-positive human serum (anti-NS3, NS4B, and NS5A), and labeled proteins were separated by SDS–10% PAGE. Note that twice the amount of immunoprecipitated sample was loaded in lanes 6 and 12. The mobilities of molecular weight standards (in thousands) are indicated on the left, and the migration of NS3, NS4B, NS5A, and 5AΔ47 is shown on the right. For data in the panel directly below the gel, total cellular RNA was extracted at 96 h posttransfection and quantified for HCV RNA levels as described in the Materials and Methods. The ratio of HCV RNA relative to the pol⁻ defective replicon is shown (HCV RNA/pol⁻). HCV RNA levels relative to the pol⁻ control were comparable in three independent experiments. For data in the bottom two panels, 96 h after transfection cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% saponin, stained for either HCV core or NS3 antigens, and analyzed by FACS. The percentage of cells expressing core and NS3 relative to an isotype matched irrelevant IgG is displayed. Values <1.5% were considered negative (−).

functional NS3 protein. However, a bicistronic derivative, where expression of ubiquitin/NS3-5B was under the control of the EMCV IRES, replicated as efficiently as SG-Neo (S2204I) RNA (data not shown), suggesting that HCV IRES-driven translation may be sensitive to RNA elements present within the core-ubiquitin coding sequence.

We also tested a replicon lacking *neo* but retaining the EMCV IRES (SG-5'HE derivatives; Fig. 1). SG-5'HE (S2204I) and SG-5'HE (5AΔ47) RNA levels relative to those in the pol⁻ control were measured 96 h after transfection and found to be higher than the selectable versions in both Huh-7.5 and Huh-7 cells (Fig. 3). Moreover, the levels of SG-5'HE (S2204I) and SG-5'HE (5AΔ47) RNA were significantly higher in Huh-7.5 compared to Huh-7 cells (Fig. 3, lanes 2, 3, 8, and 9). Approximately 52% of Huh-7.5 cells stained positive for the NS3 antigen after transfection of SG-5'HE (S2204I) RNA, compared to 21% for SG-Neo (S2204I) RNA (Fig. 3, lanes 2 and 4). Similarly, a higher frequency of Huh-7.5 cells expressed NS3 after electroporation with SG-5'HE (5AΔ47) compared to SG-Neo (5AΔ47) (11 versus 5% [Fig. 3, lanes 3 and 5]). As expected, lower frequencies of NS3-positive cells were observed for transfected Huh-7 cells (Fig. 3). The relative amounts of immunoprecipitated ^{35}S-labeled HCV proteins from Huh-7.5 and Huh-7 cells paralleled both the frequency of NS3-positive cells and relative HCV RNA levels (Fig. 3). Taken together, these data demonstrate that replicons lacking the *neo* gene initiate RNA replication more efficiently. These constructs, together with the highly permissive Huh-7.5 subline, are valuable tools for genetic studies on HCV RNA replication, some of which are described later in this report.

We next assessed the ability of Huh-7.5 cells to support replication of full-length HCV RNA containing S2204I in NS5A [FL (S2204I)] (Fig. 1). Ninety-six hours after transfection of Huh-7.5 and Huh-7 cells, the relative levels of HCV RNA and protein were measured as described above. A 50-fold increase in HCV RNA relative to that in pol⁻ cells was observed after transfection of Huh-7.5 cells, compared to only a 3-fold increase in Huh-7 cells (Fig. 3, lanes 6 and 12). Similarly, FACS analysis and immunoprecipitation of metabolically labeled proteins failed to detect HCV antigen expression in FL (S2204I) RNA-transfected Huh-7 cells, whereas 14 and 10% of Huh-7.5 cells expressed core and NS3 antigens, respectively, and ^{35}S-labeled NS3 was detectable (Fig. 3, lanes 6 and 12). The frequency of core antigen-positive cells was consis-

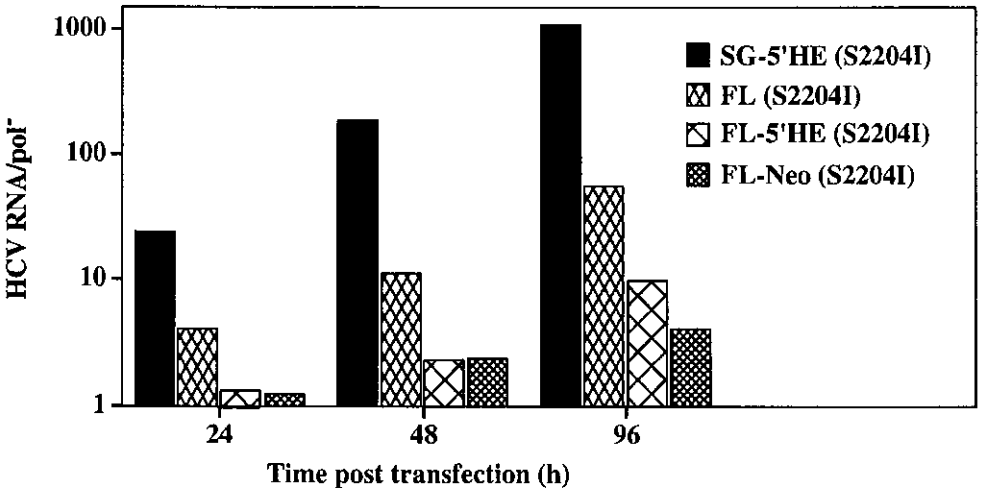

FIG. 4. HCV RNA accumulation after transfection of Huh-7.5 cells with full-length HCV RNA. One microgram of in vitro-transcribed RNA was electroporated into Huh-7.5, and 2×10^5 cells were plated into 35-mm-diameter wells. Total cellular RNA was isolated at 24, 48, and 96 h posttransfection, and HCV RNA levels were quantified as described in the Materials and Methods. The ratio of HCV RNA relative to the pol⁻ defective subgenomic RNA (HCV RNA/pol⁻) was plotted against the time posttransfection, and similar results were obtained when this experiment was repeated

tently higher than that seen for NS3, possibly reflecting differences in antibody affinity. The ability of full-length HCV RNA to establish replication in Huh-7.5 cells demonstrates that replication is not dependent upon EMCV IRES-driven translation of HCV-encoded replicase components. In fact, inclusion of the EMCV IRES downstream of the HCV 5′ NTR [FL-5′HE (S2204I)] (Fig. 1) or creation of a bicistronic construct with the *neo* gene added [FL-Neo (S2204I)] (Fig. 1) impaired replication relative to FL (S2204I) RNA (Fig. 4). It is interesting that all of the constructs containing the complete HCV coding sequence (S2204I-containing FL, FL-5′HE and FL-Neo) were less efficient at establishing replication than subgenomic replicons lacking the structural-NS2 coding region [e.g., SG-5′HE (S2204I)] (Fig. 4). This suggests that *cis* RNA elements or proteins encoded in this region of the genome may downregulate the efficiency of HCV replication in this system. Nonetheless, the ability of Huh-7.5 cells to support replication of both FL and FL-Neo RNAs provides systems that may be useful for studying steps in particle assembly and examining the impact of the entire HCV protein complement on host cell biology.

Effect(s) of mutations in NS3 and NS5A on HCV RNA replication. Besides the cellular environment, adaptive mutations selected in Huh-7 cells exhibit a wide spectrum of different efficiencies (5, 9, 15, 17). Thus far, the best single mutation we have identified was the S2204I substitution in NS5A (5). To examine the importance of Ile at this position and to see if replication efficiency could be improved further, we tested a number of other amino acids at this position and compared the replication efficiency of these replicons to SG-5′HE (S2204I) or the unmodified parent, SG-5′HE (S2204) (Fig. 5). Replicative ability was assessed in RNA-transfected Huh-7.5 cells by comparing the HCV RNA levels to that in a SG-pol⁻ control. Comparable levels of HCV RNA were observed at 96 h for replicons containing Ile or Val at position 2204, whereas an Ala substitution resulted in a threefold reduction in HCV RNA compared to SG-5′HE (S2204I) (Fig. 5). In contrast, the remaining amino acid substitutions dramatically reduced HCV RNA to levels similar to the unmodified parental replicon, SG-5′HE (S2204) (~1,400-fold decrease; Fig. 5). As expected, the relative HCV RNA levels were lower at 24 and 48 h after transfection; however, the levels were sufficient to assess replicative ability at 48 h (Fig. 5). Although substitutions that enhance subgenomic replication above that observed with SG-5′HE (S2204I) were not found, Val and Ala at position 2204 in NS5A allowed efficient RNA replication.

We investigated the replication efficiency of subgenomic replicons carrying multiple adaptive mutations in NS5A. NS5A mutations S2197P, A2199T, and S2204I independently enhance G418-resistant colony formation approximately 2,500-, 15,000-, and 20,000-fold, respectively (5). SG-5′HE replicons (Fig. 1) carrying S2204I together either with A2199T or with A2199T and S2197P were constructed, and HCV RNA levels in Huh-7.5 cells were measured by RT-PCR. Combining these NS5A mutations led to a reduction in HCV RNA levels compared to SG-5′HE (S2204I), with a 13-fold decrease for the combination of A2199T and S2204I and negligible replication when all three were combined (Fig. 6). Despite the observation that each of these NS5A adaptive mutations alone enhanced replication, when combined, the replicative ability of subgenomic RNAs declined, suggesting that these combinations are incompatible.

We previously isolated a G418-resistant cell clone harboring replicon RNA with the S2204I mutation in combination with a substitution in the protease domain of NS3 (Q1112R) (5). In light of recent observations by Krieger and colleagues (15) in which mutations in NS3 and NS5A were reported to enhance RNA replication in a synergistic manner, we examined the effect(s) of combining NS3 mutation(s) with S2204I. NS3 changes at positions 1112 (Q to R) (5), 1202 (E to G) (15), and 1280 (T to I) (15) were engineered into SG-5′HE (S2204I) (Fig. 1), and their replication in Huh-7.5 cells was determined by measuring HCV RNA levels, by measuring the frequency of

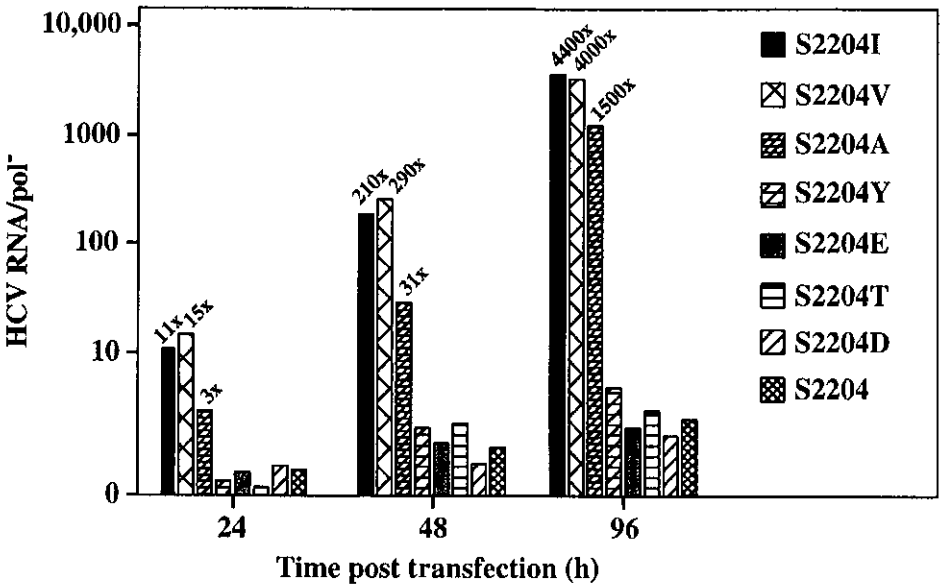

FIG. 5. Effect(s) of alternative substitutions at position 2204 in NS5A on HCV RNA replication. Huh-7.5 cells were transfected with 1 μg of the SG-5'HE replicons carrying the indicated amino acid substitutions and 2×10^5 cells plated in 35-mm-diameter wells. After 24, 48, and 96 h in culture, total cellular RNA was extracted and HCV RNA levels were measured as described in Materials and Methods. The ratio of HCV RNA relative to the pol⁻ defective subgenomic RNA (HCV RNA/pol⁻) was plotted against the time posttransfection. The increase in HCV RNA above pol⁻ is indicated above each bar. In this figure the levels of HCV RNA relative to the pol⁻ are the highest we have achieved so far. When these RNAs were transfected into Huh-7.5 cells a second time, a similar trend in HCV RNA accumulation was observed.

antigen-positive cells, and by detection of ^{35}S-labeled proteins at 96 h following transfection. Equivalent levels of HCV RNA relative to the pol⁻ RNA control were observed for each replicon (Fig. 7A). The percentages of NS5B-positive cells detected by FACS (~30%) (Fig. 7A) and immunofluorescence (Fig. 7B) were also similar. However, the frequency of NS3-positive cells was higher for replicons carrying the NS3 mutations (~73 to 87%; Fig. 7A), which may simply reflect altered affinity of the NS3-specific antibody for these NS3 mutants. Finally, the levels of immunoprecipitated NS3, NS4B, and NS5A were comparable (Fig. 7A). Although we did not verify that the Q1112R change alone was adaptive, Krieger and co-

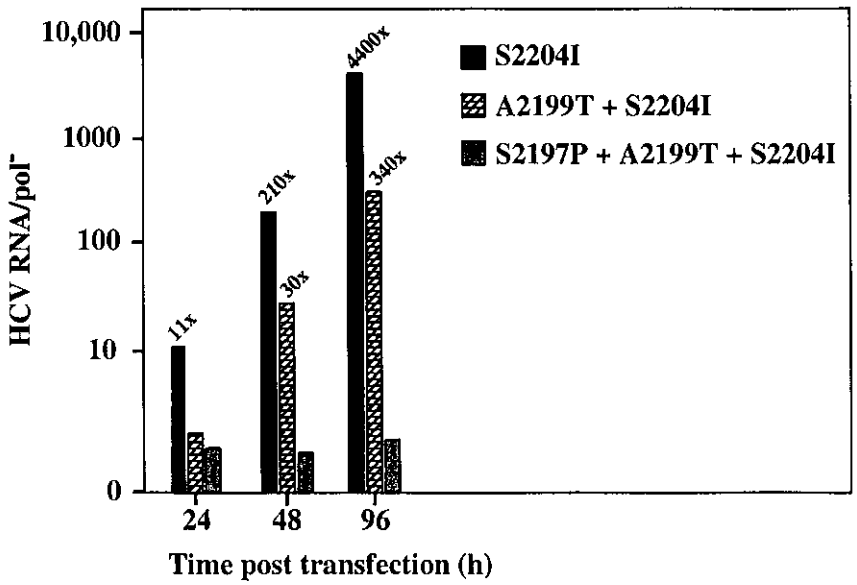

FIG. 6. Effect(s) of combining NS5A adaptive mutations on HCV RNA replication. Subgenomic replicons (SG-5'HE) carrying the indicated mutations were transfected into Huh-7.5 cells and HCV RNA levels were quantitated as described in Fig. 5. The ratio of HCV RNA relative to the pol⁻ defective subgenomic RNA (HCV RNA/pol⁻) was plotted against the time posttransfection, and the increase in HCV RNA above pol⁻ is indicated above each bar. An additional transfection experiment yielded HCV RNA/pol⁻ ratios similar to those illustrated here.

workers previously reported that the E1202G and T1280I changes alone or together increased the replication efficiency by ~13-, 6-, and 25-fold, respectively (15). Hence, these NS3 adaptive mutations do not further enhance replication when combined with the S2204I mutation in NS5A.

Mutagenesis of the S2194 NS5A phosphorylation site. The role of NS5A phosphorylation in HCV replication remains a mystery. Previously we noted differences in the extent of NS5A phosphorylation between replicons with different adaptive mutations in NS5A (5). For example, replicons with S2197C, S2197P, or S2204I expressed minimal or no p58 as assessed by one-dimensional SDS-PAGE separation of immunoprecipitated NS5A, suggesting that NS5A hyperphosphorylation is not essential for HCV replication. Recently, S2194 in NS5A of a subtype 1b isolate was identified as the primary site of p56 phosphorylation (12). To assess the possible requirement for phosphorylation of NS5A S2194, this residue was mutated in SG-Neo (S2204I) (Fig. 1) to either Ala (S2194A + S2204I) or Asp (S2194D + S2204I), to eliminate or mimic phosphorylation, respectively. G418 transduction efficiencies of these replicons in Huh-7 cells were significantly lower than those of SG-Neo (S2204I) (120- and 17-fold lower [Fig. 8A]). To rule out the possibility that G418-resistant foci were generated by reversion at this locus, the NS5A coding region was amplified from total cellular RNA by RT-PCR and directly sequenced. The original Ala and Asp substitutions at position 2194 were confirmed (data not shown). To minimize the impact of possible second-site compensating changes, we also measured HCV RNA and protein expression 96 h after RNA transfection of Huh-7.5 cells. The HCV RNA levels in replicons containing either S2194A with S2204I or S2194D with S2204I relative to those in the pol$^-$ control were ~37- and 5-fold lower than those in SG-Neo (S2204I) (Fig. 8B), consistent with their reduced ability to render Huh-7 cells G418 resistant. In addition, a lower frequency of NS5B-positive cells was evident in S2194A + S2204I than in S2194D + S2204I mutants (data not shown), and ^{35}S-labeled NS3 and NS4B were only detectable in Huh-7.5 cells transfected with SG-Neo (S2204I) and SG-Neo (S2194D + S2204I) (Fig. 8B). We were unable to directly study the phosphorylation status of NS5A expressed from cells with S2194A + S2204I and S2194D + S2204I changes since the levels of NS5A expressed in transiently transfected cells were below our detection limit (Fig. 8B and data not shown). Although the quantitative differences in G418 transduction and replication efficiencies are difficult to interpret given the possible incompatibility of combining the S2194 substitutions with the S2204I adaptive change, these data show that phosphorylation of S2194 is not an absolute requirement for HCV replication.

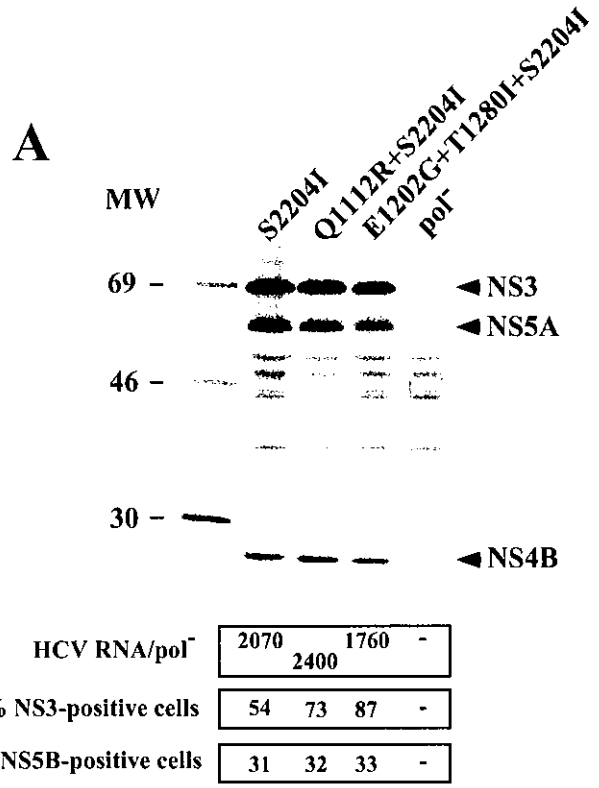

FIG. 7. Effect(s) of combining NS3 and NS5A mutations on HCV RNA replication. Subgenomic replicons lacking *neo* (SG-5'HE) were generated carrying S2204I with further mutations in NS3. (A) For the gel shown at top, 96 h after RNA transfection of Huh-7.5 cells, monolayers were labeled with ^{35}S-protein labeling mixture; cells were lysed; and NS3, NS4A, and NS5A were analyzed by immunoprecipitation, SDS–10% PAGE, and autoradiography. Positions of the molecular weight standards (in thousands) are given on the left, and HCV-specific proteins are indicated to the right. For data in the panel directly below the gel, total cellular RNA was extracted at 96 h post-transfection and HCV RNA levels were quantified as described in Materials and Methods. The ratio of HCV RNA relative to the pol$^-$ negative control is shown (HCV RNA/pol$^-$). Comparable ratios were obtained in two independent experiments. For data in the bottom two panels, 96 h after transfection, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% saponin, stained for HCV NS3 and NS5B antigens, and analyzed by FACS. The percentage of cells expressing NS3 and NS5B relative to an isotype-matched irrelevant IgG is displayed. Values <1.5% were considered negative (−). (B) Transfected cells seeded in eight-well chamber slides were fixed, permeabilized, and stained for NS5B by immunofluorescence after 96 h in culture. Nuclei were counterstained with Hoechst 33342, and stained cells visualized by fluorescence microscopy (magnification, × 40).

DISCUSSION

Several groups have demonstrated that single amino acid substitutions in the HCV replicase can dramatically increase the efficiency with which subgenomic replicons initiate replication and persist in Huh-7 cells (5, 9, 15, 17). In this study we show that Huh-7 sublines, in particular Huh-7.5 cells, possess a cellular environment that is more permissive for the initiation of HCV RNA replication. For the replicon containing the highly adaptive NS5A S2204I mutation, at least 30% of the Huh-7.5 cells can be transduced to G418 resistance. A comparable fraction was positive for the NS3 antigen by FACS. Similarly, for the SG replicons lacking *neo* (SG-5'HE in Fig. 1), at least 50% initiation efficiency was achieved. It is likely that these are low estimates, since G418 transduction efficiency is based on the number of cells used for electroporation (and not all cells survive). Indeed, more sensitive FACS analysis suggests that >75% of the cells that survive the transfection procedure harbor replicating HCV RNAs (unpublished results). While it is unclear at present how these cells will compare to the natural host cell for HCV infection, liver resident hepatocytes, they nonetheless provide a useful substrate for future genetic and biochemical studies of HCV.

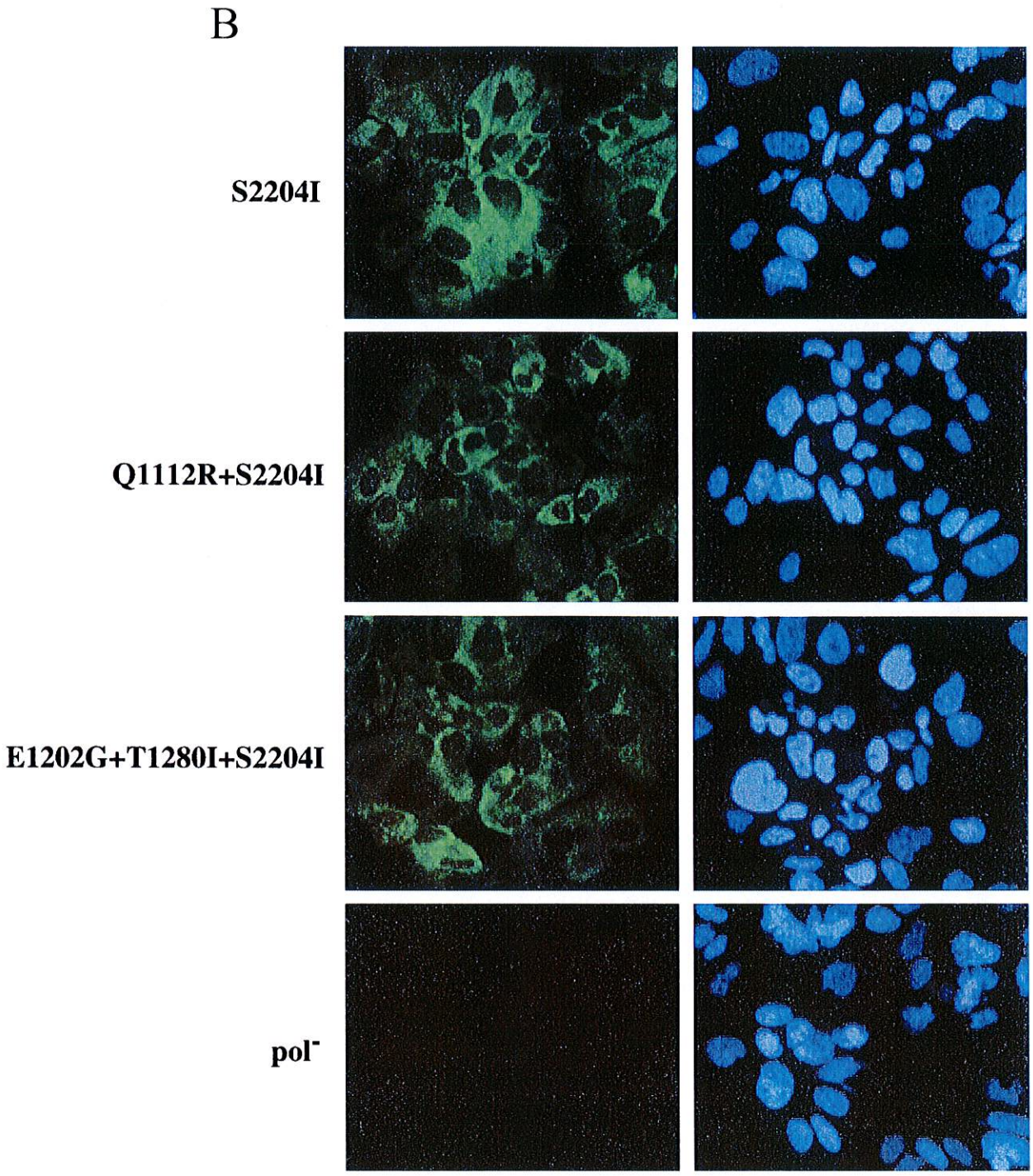

FIG. 7—*Continued.*

These highly permissive cells were obtained by curing replicon-containing cell clones with IFN-α. While we cannot rule out the possibility that IFN-α-mediated changes persist in the cellular environment, allowing Huh-7.5 cells to be more permissive for HCV replication, the fact that IFN-α treatment of parental Huh-7 cells did not alter the ability of HCV RNA to replicate suggests otherwise. Interestingly, the most highly permissive sublines (Huh-7.5 and Huh-7.8) were obtained from G418-selected clones that harbored replicons without adaptive changes in the NS3-5B region (at least at the population se-

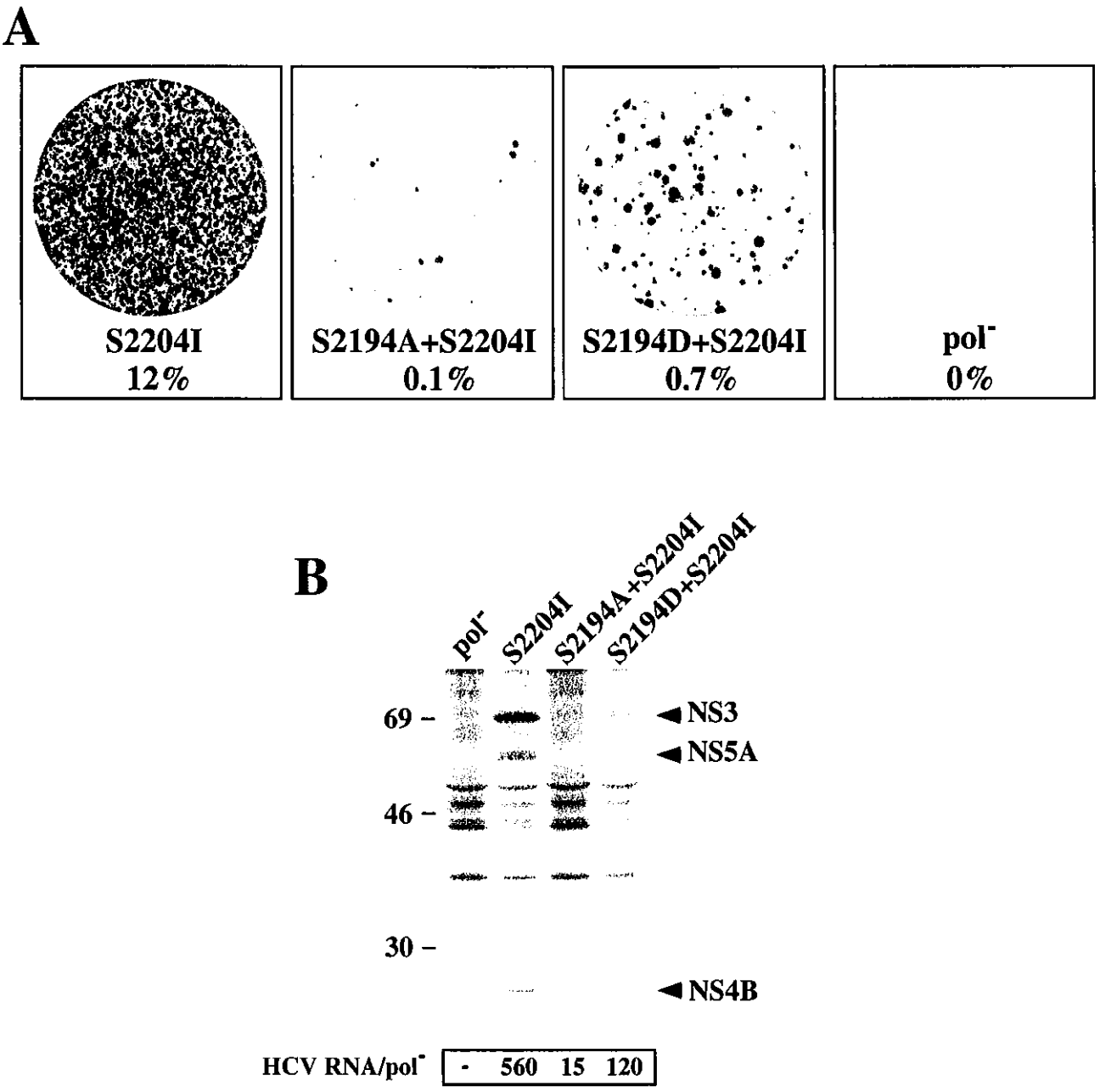

FIG. 8. Effect(s) of S2194A and S2194D mutations on HCV RNA replication. S2194 was replaced with Ala or Asp in the selectable bicistronic replicon SG-Neo (S2204I), and RNA was transcribed in vitro. (A) RNA transcripts were transfected into Huh-7 cells, and G418-selected colonies were fixed and stained with crystal violet. The relative G418 transduction efficiencies are indicated below each dish. (B) Ninety-six hours posttransfection Huh-7.5 cells were labeled with [^{35}S]methionine and [^{35}S]cysteine for 10 h. Cells were lysed, and HCV proteins were isolated by immunoprecipitation using a patient serum specific for NS3, NS4B, and NS5A. HCV proteins and the positions of protein molecular weight standards (in thousands) are shown. The ratio of HCV RNA relative to the pol$^-$ negative control at 96 h posttransfection is shown below each track (HCV RNA/pol$^-$). The results illustrated are representative of two independent transfections.

quence level). These cells may represent a subpopulation of the original Huh-7 parental line that are permissive for replication of unmodified replicons as well as more permissive for replicons with adaptive mutations. Interestingly, curing of other replicon-containing cell lines did not always yield a cell population that was more permissive for the replicons tested. For instance, curing the Huh-7 population containing the SG-Neo (S2204I) replicon, yielded a cell substrate that was unchanged in its ability to support SG-Neo (S2204I) but less efficient (23-fold) for initiation of SG-Neo (5AΔ47) (Fig. 2). These results suggest a complex interplay between the cellular environment and particular adaptive mutations to achieve productive replication.

The ability to study HCV replication directly after transfec-

tion, without the need for G418 selection, allowed us to examine replication of subgenomic replicons lacking *neo* as well as full-length HCV genome RNAs. Our initial attempt to create a monocistronic replicon by fusing cellular ubiquitin in-frame between the first 12 amino acids of core and NS3 [SG-5′Ub-NS3 (S2204I)] (Fig. 1) was unsuccessful. A bicistronic derivative with ubiquitin fused to NS3-5B was viable, suggesting that the failure of SG-5′Ub-NS3 (S2204I) to replicate was not due to a defect in processing at the ubiquitin/NS3 junction (data not shown). Rather, the fusion of the ubiquitin-coding sequence near the HCV 5′ NTR may have interfered with translation due to the formation of deleterious RNA secondary structures (20, 26) or RNA replication, by disrupting RNA elements that lie in the HCV 5′ NTR or its complement (7, 13). Fusion of the HCV 5′ NTR to the EMCV IRES yielded a subgenomic replicon (SG-5′HE) that replicated better than SG-Neo replicons (Fig. 3). Why deletion of the first cistron (sequences encoding C-Neo fusion) from the bicistronic SG-Neo (S2204I) stimulated replication is unknown, but the stimulation may result from enhanced translation of the replicase due to eliminated binding of the 40S ribosomal subunit to the usual HCV translation initiation site and diminished competition between the EMCV and HCV IRES elements (J. Marcotrigiano and C. M. Rice, unpublished results).

A similar picture was observed for replication of the full-length constructs containing the NS5A S2204I adaptive change (Fig. 4). In Huh-7.5 cells, the bicistronic construct containing the C-Neo cistron [FL-Neo (S2204I)] (Fig. 1) initiated replication less efficiently than the RNA with the HCV 5′ NTR fused to the EMCV IRES [FL-5′HE (S2204I)] (Fig. 1). Interestingly, the FL construct with the unmodified HCV genome (except for the S2204I substitution in NS5A) was better at initiating replication than RNA whose translation was mediated by the EMCV IRES (Fig. 4), demonstrating that EMCV IRES-driven translation is not required for HCV replication in Huh-7.5 cells, thus allowing the study of unmodified HCV genomic RNAs. This point could certainly impact the ability of HCV RNAs to be packaged into infectious particles. However, in our hands (Blight and Rice, unpublished results) and in a recent report (22) selective packaging of these unmodified FL RNAs was not observed in Huh-7 cells. Interestingly, full-length HCV RNAs were less efficient at establishing replication than the corresponding adapted subgenomic replicons, suggesting that addition of the structural-NS2 coding region inhibits HCV replication initiation. Whether this is due to the encoded proteins or RNA elements that lie in this region (or both) (31) is currently unclear.

In an attempt to further enhance HCV replication in cell culture, we also examined the effect of other amino acid substitutions at position 2204 in NS5A. Efficient subgenomic RNA replication was observed for Ile and Val and, to a much lesser extent, Ala at position 2204 (Fig. 5). Val or Ile are small, β-branched, nonpolar residues, whereas Ala has similar properties but is not β-branched. In contrast, polar residues such as Tyr, Glu, Thr, Ser, or Asp at position 2204 severely impaired HCV replication (Fig. 5), suggesting that replication favors nonpolar residues at this locus. It is interesting that Ser is found naturally at position 2204 for this genotype 1b isolate (18) and is conserved between other HCV genotypes (30), suggesting that this residue may be important for HCV replication and/or pathogenesis in vivo.

Combining NS5A adaptive mutations resulted in replicons that were either impaired (A2199T + S2204I) or unable to replicate (S2197P + A2199T + S2204I) in Huh-7.5 cells (Fig. 6). Incompatibility of adaptive mutations elsewhere in the HCV NS coding region has been previously described (17). For example, combining an adaptive mutation in NS5B (R2884G) with either NS4B (P1936S) or NS5A (E2163G) drastically reduced the efficiency of G418-resistant colony formation. On the other hand, combining certain NS3 and NS5A adaptive mutations can increase replication efficiency (15). However, despite the observation that mutations E1202G and T1280I in NS3 act synergistically with S2197P in NS5A to increase the replication efficiency (15; Blight and Rice, unpublished results), engineering these NS3 changes into SG-5′HE (S2204I) did not enhance replication in our system (Fig. 7). These results again underscore the empirical nature of optimizing adaptive mutations with different Huh-7 cellular environments.

The phosphorylation of NS5A is conserved among divergent HCV genotypes (25), suggesting that it plays an important role in the virus life cycle. We previously showed that NS5A hyperphosphorylation is not essential for HCV replication (5). Following the recent identification of S2194 as a major phosphate acceptor site for subtype 1b (12), we substituted Ala or Asp at this position and examined the effect on HCV replication in the context of the S2204I adaptive mutation. Given the incompatibilities observed when combining NS5A mutations, the absolute replication efficiencies of the different mutants could not be evaluated; however, replicating RNAs were recovered that harbored these substitutions at the 2194 locus. These results show that phosphorylation at S2194 is not an absolute requirement for replication of this subtype 1b isolate. While this observation deserves further study in the context of adaptive mutations outside of NS5A, it should be noted that NS5A phosphorylation is complex and only a few of the potential serine acceptor sites have been identified (12, 24). Hence, additional phosphorylation sites need to be defined and coupled with mutagenesis of these sites as well as a thorough mutational analysis of the NS5A protein.

In conclusion, we have isolated a Huh-7 subline (Huh-7.5) that is highly permissive for replication of subgenomic and full-length HCV RNAs. These cells provide a valuable substrate for future genetic studies on HCV proteins and RNA elements. Finally, Huh-7.5 and other cured Huh-7 cells that differ in their ability to support HCV replication may prove useful for defining cellular parameters that affect the efficiency of HCV RNA replication initiation by gene array and other approaches.

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