Table 2 Comparison of 1995–2000 and 2001–2006 data of 211 patients of group A

	1995–2000	2001–2006	*P*-value
Number of patients	79	132	
Sex (M/F)	44/35	80/52	$P = 0.483$
Age (median [range])	66 (49–80)	67 (49–86)	$P = 0.352$
Background (HCV/HBV/HCV[–] and HBV[–])	71/3/5	108/15/9	$P = 0.156$
Prothrombin activity (%; median [range])	79 (51–130)	82 (35–115)	$P = 0.090$
Total bilirubin (mg/dL; median [range])	1.1 (0.4–3.0)	0.9 (0.3–3.3)	$P = 0.089$
Albumin (g/dL; median [range])	3.4 (1.8–4.4)	3.7 (2.4–5.1)	$P = 0.004$
Child–Pugh class (A/B or C)	46/33	95/37	$P = 0.040$
Cirrhosis (yes/no)	69/10	105/27	$P = 0.191$
AFP (ng/mL; median [range])	20 (3–5765)	16 (1–195741)	$P = 0.129$
DCP (<100 /≥100 mAU/mL)	61/18	112/20	$P = 0.163$
Tumor size (mm; median [range])	18.0 (7–50)	18.5 (8–99)	$P = 0.406$
Tumor number (1/2–3/≥4)	48/25/6	89/36/7	$P = 0.581$
Vascular invasion (yes/no)	0/79	4/128	$P = 0.118$
Extrahepatic metastasis (yes/no)	0/79	1/131	$P = 0.438$
Milan criteria (met Milan/outside Milan)	72/7	120/12	$P = 0.955$
Treatment (HR or LAT/IVR or supportive care)	55/24	119/13	$P < 0.0001$

HCV, hepatitis C virus; HBV, hepatitis B virus; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; HR, hepatic resection; LAT, locoregional ablative therapies; IVR, interventional.

patients diagnosed during the latter period than in those detected earlier. Tumor characteristics including size, number, vascular invasion, extrahepatic metastasis and Milan criteria for HCC were comparable between the two periods. However, the frequency of receiving promising treatment was significantly higher in the latter 6 years than in the former 6 years (Table 2). For group B, significantly older age, higher frequency of HCV- and HBV-unrelated HCC and Child–Pugh class A, higher serum levels of prothrombin activity, albumin and DCP, and lower serum levels of total bilirubin and AFP were noted in the latter 6 years than in the former 6 years.

Table 3 Comparison of 1995–2000 and 2001–2006 data of 544 patients of group B

	1995–2000	2001–2006	*P* value
Number of patients	271	273	
Sex (M/F)	190/81	183/90	$P = 0.439$
Age (median [range])	66 (16–87)	69 (32–88)	$P = 0.001$
Background (HCV/HBV/HCV[–] and HBV[–])	232/29/10	222/25/26	$P = 0.022$
Prothrombin activity (%; median [range])	77 (24–130)	81 (36–122)	$P = 0.011$
Total bilirubin (mg/dL; median [range])	1.0 (0.2–7.8)	0.9 (0.3–12.5)	$P = 0.024$
Albumin (g/dL; median [range])	3.5 (2.1–4.6)	3.7 (1.8–4.8)	$P < 0.0001$
Child–Pugh class (A/B or C)	169/102	194/79	$P = 0.031$
Cirrhosis (yes/no)	222/49	205/68	$P = 0.060$
AFP (ng/mL; median [range])	52 (1–124714)	31 (2–883828)	$P = 0.001$
DCP (<100/≥100 mAU/mL)	196/75	175/98	$P = 0.040$
Tumor size (mm; median [range])	22.0 (8–105)	25.0 (9–140)	$P < 0.0001$
Tumor number (1/2–3/≥4)	122/86/63	153/80/40	$P = 0.012$
Vascular invasion (yes/no)	15/256	22/251	$P = 0.242$
Extrahepatic metastasis (yes/no)	2/269	5/268	$P = 0.258$
Milan criteria (met Milan/outside Milan)	184/87	190/83	$P = 0.669$
Treatment (HR or LAT/IVR or supportive care)	140/131	190/83	$P < 0.0001$

HCV, hepatitis C virus; HBV, hepatitis B virus; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; HR, hepatic resection; LAT, locoregional ablative therapies; IVR, interventional.

Table 4 Comparison of 1995–2000 and 2001–2006 data of 319 patients of group C

	1995–2000	2001–2006	*P* value
Number of patients	162	157	
Gender (M/F)	143/19	127/30	$P = 0.068$
Age (median [range])	65 (29–83)	64 (32–87)	$P = 0.760$
Background (HCV/HBV/HCV[–] and HBV[–])	118/28/16	96/31/30	$P = 0.037$
Prothrombin activity (%; median [range])	82 (32–130)	85 (30–120)	$P = 0.190$
Total bilirubin (mg/dL; median [range])	1.0 (0.3–7.9)	0.9 (0.1–20.0)	$P = 0.512$
Albumin (g/dL; median [range])	3.5 (2.1–4.4)	3.6 (2.1–4.6)	$P = 0.099$
Child–Pugh class (A/B or C)	112/50	116/41	$P = 0.348$
Cirrhosis (yes/no)	106/56	107/50	$P = 0.636$
AFP (ng/mL; median [range])	78 (2–976554)	72 (1–2397149)	$P = 0.877$
DCP (<100/≥100 mAU/mL)	53/109	57/100	$P = 0.500$
Tumor size (mm; median [range])	50.0 (9–180)	51.0 (10–300)	$P = 0.363$
Tumor number (1/2–3/≥4)	39/48/75	42/39/76	$P = 0.616$
Vascular invasion (yes/no)	38/124	49/108	$P = 0.120$
Extrahepatic metastasis (yes/no)	18/144	18/139	$P = 0.920$
Milan criteria (met Milan/outside Milan)	43/119	41/116	$P = 0.931$
Treatment (HR or LAT/IVR or supportive care)	43/119	46/111	$P = 0.583$

HCV, hepatitis C virus; HBV, hepatitis B virus; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; HR, hepatic resection; LAT, locoregional ablative therapies; IVR, interventional.

Tumor characteristics were contradictory, with significantly larger size tumors, but smaller numbers of HCC detected in the latter 6 years than in the former 6 years. The frequencies of vascular invasion, extrahepatic metastasis and Milan criteria for HCC were not different between the two periods. Finally, the frequency of receiving promising treatment was significantly higher in the latter 6 years than in the former 6 years (Table 3). For group C patients, liver function tests, Child–Pugh class, tumor characteristics, Milan criteria for HCC and treatment of HCC were comparable between the two periods; the only difference was a higher frequency of HCV- and HBV-unrelated HCC in the latter 6 years than in the former 6 years (Table 4).

Comparison of LAT between 1995 and 2000 and 2001–2006

Locoregional ablative therapies were used to treat 196 and 262 patients in 1995–2000 and 2001–2006, respectively. In the former 6 years, 140 (72%; 37 of group A, 85 of group B and 18 of group C), 32 (16%; six of group A, 24 of group B and two of group C) and 24 (12%; five of group A, 12 of group B and seven of group C) patients were treated with PEI, RFA and MCT, respectively. In the latter 6 years, none of the patients were treated with MCT, while 18 (7%; 11 of group A, six of group B and one of group C) and 244 (93%; 88 of group A, 138 of group B and 18 of group C) patients were treated with PEI and RFA, respectively. The frequency of receiving RFA was significantly higher in the latter 6 years than in the former 6 years.

Comparison of survival rates between 1995–2000 and 2001–2006

The cumulative survival rates between the two periods according to the manner of HCC detection are shown in Figures 1 and 2. For the surveillance (+) group (groups A and B, 755 patients), the 2-, 3- and 4-year cumulative survival rates were 83%, 71% and 58% for the latter 6 years (405 patients) and 75%, 61% and 50% for the former 6 years (350 patients), respectively (Fig. 1). The cumulative survival rates of those patients in whom HCC was detected in the latter 6 years tended to be better than those diagnosed during the former 6 years. For the surveillance (–) group (group C), the cumulative survival rates were not different between the two periods ($P = 0.5546$) (Fig. 2).

DISCUSSION

OUR STUDY WAS designed to evaluate the effect of recent improvement in management of HCC according to the method applied for detection of HCC (group A, surveillance at Kurume University School of Medicine; group B, surveillance at other institutions; group C, control group).

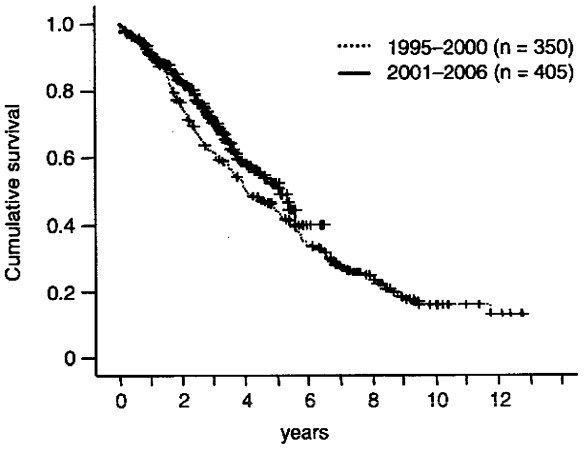

Figure 1 Kaplan–Meier survival curves of 755 patients with hepatocellular carcinoma detected by surveillance (groups A and B) in 1995–2000 and 2001–2006. The cumulative survival rate of those patients diagnosed in the latter 6 years (2001–2006) was significantly better than those diagnosed during the former 6 years (1995–2000) ($P = 0.0349$).

Sangiovanni *et al.*[7] reported that in the last quinquennium of 1987–2001, survival of HCC patients during surveillance increased as a consequence of improved early detection of the cancers,[4–13] wider application of radical therapies to accurately selected patients[2,16–23] and efficient management of liver-disease complications.[2,24,25] In the present study, improvement of surveillance did not translate into early detection of HCC in the latter 6 years (2000–2006) compared with the former 6 years (1995–2000) in either surveillance group (A or B; Tables 2,3). Surveillance for HCC based on US and AFP determination may have limited value in early detection of HCC despite the intense surveillance program in Japan. However, the frequency of patients with Child–Pugh class A and those receiving promising treatment increased more in the latter 6 years than in the former 6 years. Furthermore, the cumulative survival rates in surveillance groups in the latter 6 years tended to be better than those for patients from the former period (4-year, 58% vs 50%; Fig. 1). In Group C (control group), hepatic reserve capacity, tumor characteristics, receiving promising treatment and cumulative survival were not different between the two periods (Table 4, Fig. 2).

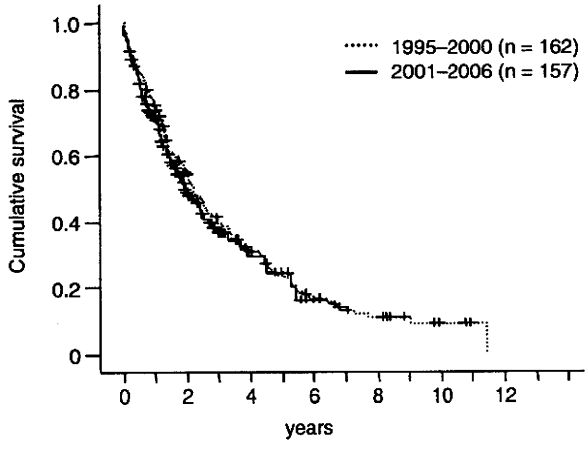

Figure 2 Kaplan–Meier survival curves of 319 patients with hepatocellular carcinoma detected incidentally or because of symptoms. The cumulative survival rates were comparable between the two periods ($P = 0.5546$).

Based on recent technological improvements in LAT, RFA is superior to PEI with regard to the achievement of complete tumor necrosis and increase in the survival chance of patients with early-stage HCC.[22,23] In the present study, the proportion of patients receiving RFA was significantly higher in the latter period compared to the earlier one. The change from PEI to RFA in the LAT treatment contributed to this improved survival in patients with HCC detected during surveillance. Moreover, Kurume University School of Medicine provided IFN therapy for patients with cirrhosis, in addition to follow up after curative treatment of HCC, and management of cirrhosis-related complications by nutritional therapists in the latter 6 years. Proper management of cirrhosis complications including IFN therapy for patients with cirrhosis and patients with HCC following promising treatment[26,27] and nutrition therapy provided by nutritional therapists[24,25] could have contributed to the increased hepatic reserve capacity and possibly survival of patients with HCC detected during surveillance.

Ultrasonography and AFP determination every 6 months for cirrhotic patients is a convenient and cost-effective surveillance program.[7–12,14–16] In the present study, US and AFP determinations were performed every 3 months for patients with chronic liver disease (including chronic hepatitis and cirrhosis). The surveillance program in the present study may be too intensive and not as cost effective. However, the median tumor size of group A was only 18.0 mm and 192 of the 211 patients (91%) met the Milan criteria for HCC. Recent progress in available treatments for early-stage HCC and in the management of cirrhosis have made such surveillance programs more important for the early detection of HCC. Randomized prospective trials are needed to determine whether surveillance for HCC can improve survival of patients with chronic liver disease.

In conclusion, patients with HCC detected in the last 6 years through surveillance were more likely to receive promising treatment and to have better prognoses than similar patients identified in 1995–2000.

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APPENDIX

Table A1 Clinical profile of 1074 patients with hepatocellular carcinoma

	Group A	Group B	Group C	Kruskal–Wallis test
Number of patients	211	544	319	
Sex (M/F)	124/87	373/171	270/49	
		$P = 0.011$	$P < 0.0001$	
			$P < 0.0001$†	
Age (median [range])	67 (49–86)	67 (16–88)	64 (29–87)	
		$P > 0.05$	$P < 0.05$	$P < 0.0001$
			$P < 0.05$†	
Background (HCV/HBV/HCV[−] and HBV[−])	179/18/14	454/54/36	214/59/46	
		$P = 0.842$	$P < 0.0001$	
			$P < 0.0001$†	
Prothrombin activity (%; median [range])	81 (35–130)	79 (24–130)	83 (30–130)	
		$P > 0.05$	$P > 0.05$	$P = 0.005$
			$P < 0.05$†	
Total bilirubin (mg/dL; median [range])	1.0 (0.3–3.3)	1.0 (0.2–12.5)	1.0 (0.1–20.0)	
		$P > 0.05$	$P > 0.05$	$P = 0.761$
			$P > 0.05$†	
Albumin (g/dL; median [range])	3.6 (1.8–5.1)	3.5 (1.8–4.8)	3.5 (2.1–4.6)	
		$P > 0.05$	$P > 0.05$	$P = 0.953$
			$P > 0.05$†	
Child–Pugh class (A/B or C)	141/70	363/181	228/91	
		$P = 0.980$	$P = 0.255$	
			$P = 0.147$†	
Cirrhosis (yes/no)	174/37	427/117	213/106	
		$P = 0.224$	$P < 0.0001$	
			$P < 0.0001$†	
AFP (ng/mL; median [range])	17 (1–195741)	39 (1–883828)	72 (1–2397149)	
		$P < 0.05$	$P < 0.05$	$P < 0.0001$
			$P < 0.05$†	
DCP (<100/≥100 mAU/mL)	173/38	371/173	110/209	
		$P < 0.0001$	$P < 0.0001$	
			$P < 0.0001$†	
Tumor size (mm; median [range])	18.0 (7–99)	24.0 (8–140)	50.0 (9–300)	
		$P < 0.05$	$P < 0.05$	$P < 0.0001$
			$P < 0.05$†	
Tumor number (1/2–3/≥4)	137/61/13	275/166/103	81/87/151	
		$P < 0.0001$	$P < 0.0001$	
			$P < 0.0001$†	
Vascular invasion (yes/no)	4/207	37/507	87/232	
		$P = 0.008$	$P < 0.0001$	
			$P < 0.0001$†	
Extrahepatic metastasis (yes/no)	1/210	7/537	36/283	
		$P = 0.328$	$P < 0.0001$	
			$P < 0.0001$†	
Milan criteria (met Milan/outside Milan)	192/19	374/170	84/235	
		$P < 0.0001$	$P < 0.0001$	
			$P < 0.0001$†	
Treatment (HR or LAT/IVR or Supportive care)	174/37	330/214	89/230	
		$P < 0.0001$	$P < 0.0001$	
			$P < 0.0001$†	

†Group B vs group C.
HCV, hepatitis C virus; HBV, hepatitis B virus; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; HR, hepatic resection; LAT, locoregional ablative therapies; IVR, interventional.

AP&T Alimentary Pharmacology and Therapeutics

Intra-arterial therapy with cisplatin suspension in lipiodol and 5-fluorouracil for hepatocellular carcinoma with portal vein tumour thrombosis

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SUMMARY

Background
Portal vein tumour thrombosis is a negative prognostic factor for hepatocellular carcinoma (HCC).

Aim
To assess the efficacy of cisplatin in lipiodol emulsion combined with 5-fluorouracil (5-FU) for patients with HCC and portal vein tumour thrombosis.

Methods
The study subjects were 51 patients with the above-specified criteria who received injection of cisplatin suspension in lipiodol emulsion followed by intra-arterial infusion of 5-FU. The primary objective was to determine tumour response to the treatment, while the secondary objectives were safety and tolerability. Independent factors for survival were also assessed.

Results
Ten patients had complete response and 34 patients had partial response (response rate, 86.3%). The median survival for all 51 patients was 33 months, while that for 10 complete response patients and 21 patients who showed disappearance of HCC following additional therapies was 39 months. The single factor that significantly influenced survival was therapeutic effect. Treatment was well tolerated and severe toxicity was infrequent, with only grade 3 toxicity (thrombocytopenia) in one patient.

Conclusions
The present study demonstrated the efficacy of hepatic arterial infusion chemotherapy using cisplatin-lipiodol emulsion and 5-FU without serious adverse effects in patients with unresectable HCC and portal vein tumour thrombosis.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is increasing worldwide and is one of the most common malignant tumours in the tropics and the Far East, including Japan.[1] It is the sixth most common cancer worldwide with 626 000 new cases in 2002.[2] HCC develops multifocally in chronically damaged liver. Epidemiological studies from Japan indicate that approximately 75% of HCC are caused by hepatitis C virus (HCV) infection and 10% by hepatitis B virus (HBV) infection.[3]

The development of sophisticated diagnostic modalities such as computed tomography (CT), magnetic resonance imaging (MRI) and abdominal ultrasonography (US), have allowed early diagnosis of HCC. Patients with small HCC are usually treated with surgical resection, liver transplantation, percutaneous ethanol injection therapy, microwave coagulation therapy, or percutaneous radiofrequency ablation. The prognosis of patients with small HCC has improved following the application of these therapeutic modalities.[4]

On the other hand, treatment of advanced HCC includes trans-hepatic arterial chemoembolization (TACE), trans-hepatic arterial infusion chemotherapy (HAIC), systemic chemotherapy, hormonal therapy and immunotherapy. However, only TACE has been confirmed to improve long-term survival.[5] In advanced HCC, tumour cells easily invade the portal vein.[6] Unfortunately, despite the progress in diagnostic techniques for HCC, portal vein tumour invasion is found in 12.5–39.7% of patients with HCC.[7–9] Portal vein tumour invasion is a crucial factor in the prognosis of patients with HCC.[10] Many clinical trials for advanced HCC with portal vein tumour thrombosis have been conducted. However, two systemic reviews confirmed negative outcome of these clinical trials.[6, 11] Two recent phase III clinical trials have shown that sorafenib, an orally available multikinase inhibitor, improves the median overall survival in patients with advanced HCC.[12, 13] Sorafenib has antivascular properties through targeting vascular endothelial growth factor (VEGF) receptor 2 and platelet-derived growth factor (PDGF) receptor and also blocks tumour cell proliferation by targeting the Raf/MEK/ERK signalling pathway.[14] However, patients with HCC and portal vein tumour thrombosis usually have very short survival and grave prognosis even when treated with sorafenib.[15] In Japan, such patients have been sometimes treated with HAIC with cisplatin and 5-fluorouracil (5-FU) or 5-FU and subcutaneous interferon-α injection.[16–19]

In the present study, we investigated the efficacy and safety of the new combination therapy of cisplatin-lipiodol suspension and 5-FU for HCC with portal vein tumour thrombosis.

PATIENTS AND METHODS

Criteria for treatment

The following criteria were used for the use of cisplatin-lipiodol suspension and 5-FU: (i) tumour thrombosis invading the portal vein (Vp2–4), (ii) absence of extrahepatic metastases, (iii) patients age >20 years, (iv) estimated life expectancy >3 months, (v) platelet count >50 000/μL and leucocyte count >2000/μL, (vi) Child-Pugh class A or class B, and (vii) performance status [Eastern Cooperative Oncology Group (ECOG)] level[20] of 0–2. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the ethics review committees of Yame Republic Hospital and Kurume University, School of Medicine. Informed written consent was obtained from each patient before enrolment in the study.

Patients

From July 2004 to February 2009, 61 consecutive patients with non-resectable HCC and portal vein tumour thrombosis (Vp2–4) were referred to Yame Republic Hospital. All patients were classified as Barcelona Clinic Liver Cancer (BCLC) stage C.[21] Each patient underwent clinical examination, US, CT, MRI, and angiography. Extrahepatic metastases were found in seven patients and three patients were Child-Pugh class C. These 10 patients were excluded from the study. We investigated the efficacy and safety of cisplatin-lipiodol plus 5-FU therapy in the remaining 51 patients (men; 43, women; 8, age 57–85 years). All patients had liver cirrhosis (Child-Pugh class A; 26, class B; 25). Thirty-nine patients were HCV antibody-positive, six were HBs antigen-positive and six were HCV antibody-negative and HBs antigen-negative. The ECOG performance status of the 51 patients was 0 or 1. These patients were free of uncontrolled ascites and hepatic encephalopathy. Leucocyte and platelet counts were >3000/μL and 50 000/μL respectively. Serum creatinine level was <1.5 mg/dL (Table 1).

Catheter placement

After local anaesthesia, a J-shaped 4-French catheter through a 4-French introducer sheath was inserted through the femoral or brachial artery by the Seldinger method. The catheter was advanced into the target artery under fluoroscopic guidance, and visceral arteriography

Table 1 | Baseline clinical characteristics

No. of patients	51
Age (years)	68.5 ± 9.2
Male/Female	43/8
Alcohol intake (+/−)	18/33
Albumin (g/dL)	3.6 ± 0.5
Total bilirubin (mg/dL)	1.2 ± 0.7
Prothrombin time (INR)	1.2 ± 0.1
White cell count (/L)	4963 + 1894
Haemoglobin (g/dL)	12.6 ± 2.2
Platelet ($\times 10^9$/L)	150 ± 86
Chiid-Pugh class : A/B	26/25
HBV (+)/HCV (+)/HBV (−) and HCV (−)	6/39/6
Previous treatment (+/−)	35/16
Portal vein invasion (trunk/first branch/second branch)	11/18/22
Maximum tumour size (mm), (<100 mm/≥100 mm)	88.6 ± 32.1, 31/20
AFP (ng/mL) (≤1000/>1000, ≤10 000/>10 000)	29/10/12
AFP L3 (≥10%(%))	86.3%
DCP (mAU/mL) (≤1000/>1000, ≤10 000/>10 000)	14/20/17
Macroscopic finding (nodular/infiltrative)	15/36
Tumour location (unilobular/bilobular)	18/33

was performed to detect HCC. The right gastric artery and gastro-duodenal artery were embolized using microcoils (Diamond Coli, Boston Scientific; Trufill, Cordis; or Hilal Embolization Microcoils, Cook Europe) to prevent gastroduodenal injury by anti-cancer agents. A polyurethane-covered catheter (Anthron P-U Catheter, Toray Medical, Tokyo, Japan) was used as the indwelling catheter. The tip of the catheter was placed in the common hepatic artery or proper hepatic artery. The other end of the catheter was connected to the injection port and the device was implanted in a subcutaneous pocket. To prevent obstruction of the catheter, 5 mL (5000 U) of heparin solution was injected biweekly via the injection port.

Treatment protocol

The cisplatin-lipiodol plus 5-FU regimen comprised a combination of 50 mg cisplatin in 5–10 mL lipiodol and continuous infusion of 5-FU (1500 mg/5 days). At day 1 of treatment, cisplatin with lipiodol was injected through the reservoir catheter followed by 5-FU (250 mg). Then, 5-FU (1250 mg) was continuously infused using a balloon pump (SUREFUSER PUMP, Nipro Pharma Corporation, Osaka, Japan) for 5 days. This regimen was applied once a week during the first 2 weeks of admission, then the combination of 20 mg cisplatin with lipiodol and 5-FU (500–1250 mg) was infused every 2 weeks at the out-patient department (OPD) as long as possible. Chemotherapy was discontinued when adverse effects reached level 2 of the ECOG classification with the exception of platelet and leucocyte counts of<30 000/μL and 2000/μL respectively.

Assessment of response to chemotherapy

The primary efficacy endpoint was objective tumour response, while the secondary endpoint was patient survival. The primary efficacy endpoint was assessed at 3 months after the initial treatment and then every 2 months. At 3 months after initial treatment, partial responders and complete responders were distinguished. Tumours were bi-dimensionally measured by dynamic CT or dynamic MRI. The response to treatment was evaluated according to the Response Evaluation Criteria in Solid Tumours (RECIST)[22] and following the EASL[23] amendments that take into account the amount of necrotic tumour: as complete response (CR), all measureable lesions disappeared for more than 4 weeks; partial response (PR), sum longest diameter decrease more than 30% and no new lesion for more than 4 weeks; progressive disease (PD), sum longest diameter increase more than 25% or appearance of new lesion; stable disease (SD), no definition of PR and PD for more than 8 weeks.

Assessment of tolerability

Safety was assessed at each study visit, by adverse events, a brief physical examination, vital sign measurements and clinical laboratory evaluation. The severity of any toxicity was assessed according to the National Cancer Institute Common Toxicity Criteria, version 3. The presence of seven clinical symptoms and signs commonly noted in patients with HCC (ascites, anorexia, jaundice, local pain, lack of energy, malaise or bodily discomfort-fatigue and intratumoural haemorrhage) and complications associated with indwelling catheter (e.g. gastro-duodenal ulcer, infection, thrombosis and vascular damage) were also assessed.

Statistical analysis

Baseline data were expressed as mean ± s.d. or as median and range values. Survival was confirmed up to

31 August 2009. Cumulative survival was calculated using the Kaplan–Meier method and compared by the log rank test. Independent factors for survival were assessed with the Cox proportional hazard regression model. Statistical significance was defined as a P value less than 0.05. The SPSS software version 14.0J (SPSS inc., Chicago, IL, USA) was used for statistical analysis.

RESULTS

Tumour characteristics

All patients were followed-up for more than 6 months. Tumour thrombosis was noted in the main portal vein in 10 patients, in the first branch in 18, and in the 2nd branch in 23 patients. The mean diameter of the main tumour was 87.0 mm (range, 50–170 mm). Serum α-fetoprotein (AFP) levels in 41 patients were >20 ng/mL. AFP-L3 was positive (>10%) in 32 patients, and 44 patients were des-γ-carboxy prothrombin (DCP)-positive (>40 AU/mL). Patients received 2–28 (median, 8.7) courses of cisplatin-lipiodol plus 5-FU therapy (Table 1).

Response to cisplatin-lipiodol plus 5-FU therapy and additional therapy

Of the 51 patients treated with this regimen, 10 (19.6%), 34 (66.7%) and 5 (9.8%) patients had a CR, PR and SD respectively [response rate (CR + PR/51) = 86.3%]. The remaining patient had PD. Of the 34 patients with PR, 24 were treated with surgical resection, RFA or TACE and showed the disappearance of visible HCC.

Survival and causes of death

Figure 1 shows the cumulative survival rates of 51 patients. The 12-, 24- and 36-month survival rates for the 51 patients were 72.9%, 58.1% and 34.9% respectively. The median survival rate of these patients was 33 (range, 3–51) months. The median survival time of CR, PR and SD patients were 39 (range, 13–51) months, 31 (range, 6–48) months and 7 (range, 4–23) months respectively. There was a significant difference in the survival time of the three groups. Figure 2 shows the cumulative survival rates of CR and PR patients, and SD and PD patients. The 12-, 24- and 36-month survival rates of the CR and PR patients were 78.4%, 61.8% and 37.1% respectively. There was a significant difference in the survival time between CR and PR patients and SD and PD patients. Figure 3 shows the cumulative survival rates of 10 patients with CR and 14 patients with PR who later showed disappearance of viable HCC after additional therapy and the remaining 27 patients who failed to be

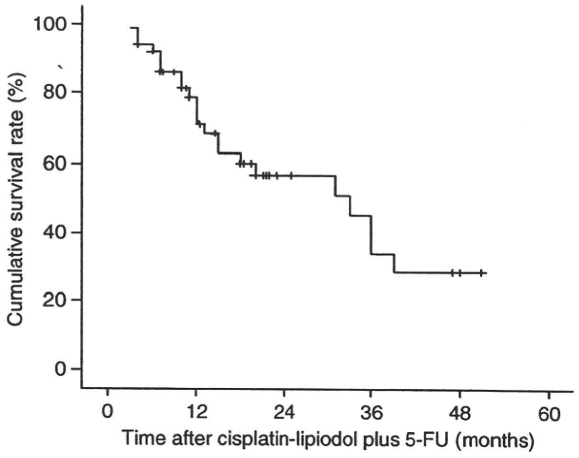

Figure 1 | Overall survival of all treated patients (n = 51).

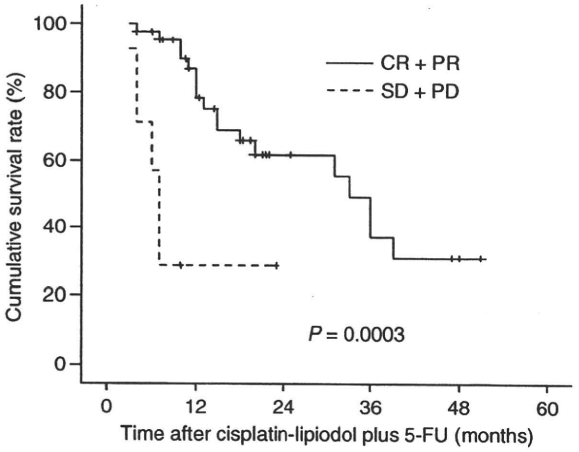

Figure 2 | Overall survival of patients who showed CR or PR and patients who showed SD or PD. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease. P = 0.0003 by Log Rank test.

tumour-free after additional therapy. The median survival time for the 24 patients who showed disappearance of viable HCC was 39 (range, 6–51) months. The 1-, 2- and 3-year survival rates of these patients were 100%, 89.5% and 53.7% respectively. On the other hand, the median survival time and 1-, 2- and 3-year survival rates of the remaining 27 patients were 12 (range, 3–25) months, and 44.8%, 24.0% and 0% respectively. There was a significant difference in survival between patients who showed disappearance of viable HCC and those with visible HCC during the treatment. Figure 4 displays the cumulative tumour progression-free survival time. The 6-, 12- and 24-month progression-free survival

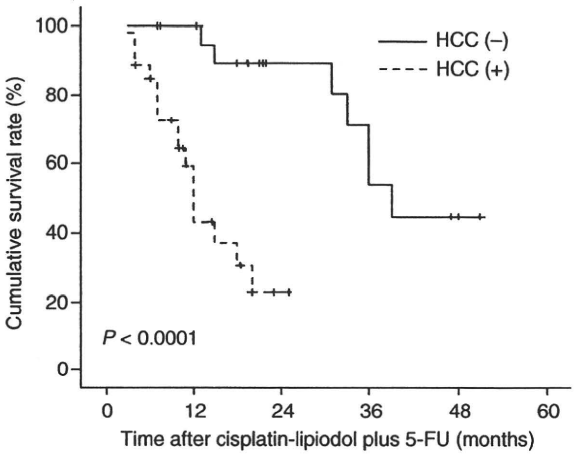

Figure 3 | Overall survival of patients who showed CR (n = 10) and disappearance of HCC after additional treatment (n = 14), and patients with variable HCC (n = 27). P = 0.0001 by Log Rank test; HCC (–), patients without variable HCC after treatment; HCC (+), patients with variable HCC after treatment; CR, complete response.

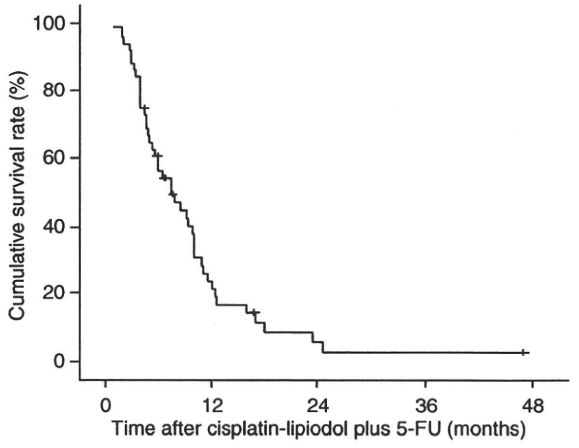

Figure 4 | Progression-free survival of all treated patients (n = 51).

rates of these patients were 62.1%, 21.9% and 11.7% respectively, with a median progression-free survival of 8.0 months. With regard to the relationship between survival and degree of tumour progression, there was no significant difference in median survival among patients with tumour thrombosis in the 2nd branches, the 1st branches and the portal vein trunk. With regard to the relationship between survival and liver damage, there was no significant difference in the median survival between patients with Child-Pugh class A and those with class B. During the follow-up period, 28 patients died. Of these, 24 died of tumour progression; two of rupture of oesophageal varices, one of liver failure and one patient died of renal failure.

Two of the 13 factors analysed by univariate analysis showed prognostic significance: tumour location (P = 0.048) and therapeutic effect (P < 0.001). Multivariate analysis identified only one variable, therapeutic effect, to be an independent predictor of mortality (P < 0.001) (Table 2).

Regarding the therapeutic effect, two of the 13 factors analysed by univariate analysis showed the therapeutic significance: tumour location (P = 0.042) and grade of portal vein invasion (P = 0.002). Multivariate analysis identified only one variable, grade of portal vein invasion, to be an independent predictor of therapeutic effect (P = 0.006) (Table 3).

Adverse effects and complications

No serious complications due to indwelling catheters, such as peptic ulcer, infection, thrombus and other vascular disorders, were observed. Treatment was not dis-

Table 2 | Univariate and multivariate analyses of survival for hepatocellular carcinoma

	HR (95% CI)	P value
Univariate analysis		
Gender (male)	1.24 (0.54-2.87)	0.609
Age (>65)	1.08 (0.32-3.65)	0.899
Alcohol intake (+)	1.15 (0.49-2.66)	0.752
HCV (positive)	0.50 (0.20-1.29)	0.152
HBV (positive)	2.03 (0.56-6.23)	0.218
Child-Pugh class (B)	1.12 (0.49-2.57)	0.786
AFP(ng/mL) (>1000)	1.51 (0.67-3.43)	0.322
DCP (AU/mL)(>1000)	1.03 (0.41-2.63)	0.946
Maximum tumour size (mm) (>100)	0.82 (0.34-2.01)	0.667
Macroscopic finding (infiltrative)	0.95 (0.39-2.31)	0.901
Tumour location (bilobular)	2.56 (1.01-6.48)	0.048
Grade of portal vein invasion (trunk)	1.62 (0.60-4.39)	0.344
Therapeutic effect (CR+PR)	0.17 (0.06-0.51)	0.001
Multivariate analysis		
Therapeutic effect (CR+PR)	0.21 (0.07-0.66)	0.007

Table 3 | Univariate and multivariate analyses of therapeutic effect for hepatocellular carcinoma (CR+PR vs. SD+PD)

	Univariate analysis		
	CR+PR group (n = 44)	SD+PD group (n = 7)	P value
Gender (male/female)	38/6	6/1	0.963
Age (≤65/>65)	18/26	3/4	0.923
Alcohol intake (+/−)	16/28	2/5	0.689
HCV (+/−)	32/12	7/0	0.177
HBV (+/−)	6/38	0/7	0.578
Child-Pugh class (A/B)	23/21	3/4	0.703
AFP (ng/mL) (≤1000/1000-10 000/≥10 000)	26/9/9	3/1/3	0.431
DCP (AU/mL) (≤1000/1000-10 000/≥10 000)	14/16/14	0/4/3	0.211
Previous treatment (+/−)	14/30	1/6	0.658
Maximum tumour size (mm) (<100 mm/≥100 mm)	28/16	3/4	0.411
Macroscopic finding (nodular/infiltrative)	31/13	4/3	0.664
Tumour location (unilobular/bilobular)	18/26	0/7	0.042
Grade of portal vein invasion (trunk/first branch/second branch)	6/16/22	5/2/0	0.002

	Multivariate analysis		
	Hazard ratio	(95% CI)	P value
Grade of portal vein invasion	0.105	(0.02-0.52)	0.006

continued for serious complications. However, only one patient developed grade 3 thrombocytopaenia. Grade 1 appetite loss was noted in 17 patients, six patients developed grade 1 high fever and two developed grade 2 ascetic fluid accumulation. These adverse effects were controlled by medical treatment.

DISCUSSION

The prognosis of HCC patients with portal vein tumour thrombosis is very poor. Portal vein tumour thrombosis is a significant clinicopathological variable known to influence survival of patients with advanced HCC.[24] The median survival of untreated patients with HCC and portal vein tumour thrombosis is reported to be 2.7–4.0 months, whereas the survival of those without portal vein tumour thrombus is 24.4 months.[25, 26] To improve this bleak outcome, various treatments have been applied. Takizawa *et al.*[24] reported that the longest median survival (26.0 months) was associated with surgical resection, followed by continuous HAIC (8.0 months) and transcatheter arterial infusion/TAE (5.5 months). However, the number of patients with HCC and portal vein tumour thrombosis who are suitable for surgical resection is limited. In such situation, systemic chemotherapy, hormonal therapy and radiotherapy have all been reported to be of limited value.[27] Recently, two phase 3, double-blind, placebo-controlled trials designed to assess the efficacy of sorafenib for patients with advanced HCC were conducted.[12, 13] They demonstrated prolonged overall survival and time to progression in sorafenib-treated patients. Llovet *et al.*[21] propose the use of sorafenib as the 1st line treatment for patients with HCC categorized as BCLC C. However, in the SHARP trial, the median overall survival time of patients treated with sorafenib was 10.7 months.[12] In another study, the median overall survival of patients with portal vein tumour thrombosis was only 5 months.[15]

Trans-hepatic arterial infusion chemotherapy is a reasonable drug delivery system for patients with advanced HCC because advanced HCC receives most of its blood supply from the hepatic artery and non-cancerous liver is supplied mainly by the portal vein.[28] HAIC seems to deliver high concentrations of chemotherapeutic agents to HCC tissues selectively with low toxicity to non-cancerous liver tissues and whole body. Several reports described the effects of HAIC with cisplatin and 5-FU or systemic interferon-α therapy with HAIC using 5-FU for HCC patients with tumour thrombosis in the 1st branches and the portal

vein trunks. Although the response rate varied from 0% to 63%, the median survival time was less than 11.8 months.[16, 17, 19, 29–31] We also reported previously the efficacy of HAIC with cisplatin and 5-FU for HCC patients with portal vein tumour thrombosis. In that study, the response rate and median survival time were 48% and 10.2 months respectively.[17] Recently, Salem *et al.*[32] treated HCC patients with intra-arterial yttrium-90 microspheres. In their report, the median overall survival of patients with Child-Pugh class A and that of patients with class B who had portal vein tumour thrombosis were 10.4 and 5.6 months respectively. In the present study, the response rate was 86.3%. The degree of portal vein tumour thrombosis was a predictor of treatment effect. The overall survival rates at 12, 24 and 36 months were 72.9%, 58.1% and 34.9% respectively, and the overall median survival rate was 33 months. Univariate and multivariate analyses showed that only the therapeutic effect was an independent prognostic factor of survival. To the best of our knowledge, cisplatin-lipiodol plus 5-FU therapy is associated with the longest survival of HCC patients with portal vein tumour thrombosis in all studies reported so far. The rationale of cisplatin+5-FU regimen is that cisplatin and 5-FU have antitumour effects;[33] cisplatin has a synergistic effect as a modulator of 5-FU.[34] In HAIC with cisplatin and 5-FU or IFN and 5-FU, 5-FU is usually infused for 3–5 h.[17, 19, 30] In the present study, 5-FU was continuously infused for 5 days. 5-FU does not show a dose-dependent, but time-dependent antitumour effect.[35] Continuous infusion of 5-FU might enhance the antitumour effect in cisplatin-lipiodol plus 5-FU therapy compared with other HAICs. Anticancer agents in lipiodol suspension are reported to exhibit a more potent antitumour effect than anticancer agent alone.[36] Two possible mechanisms may explain this enhanced effect; embolization of tumour artery by lipiodol, and retention and continuous release in tumour tissues of anticancer agent. In the present study, a high concentration of cisplatin (9.8–13.5 $\mu g/g$ wet tissue weight) was detected in the resected tumour tissues after 132 days of cisplatin with lipiodol injection. Furthermore, the concentration of cisplatin in tumour tissues was higher than that in adjacent non-tumour tissues (data not shown). The reason for the long median survival of patients participating in the present study could be the continuous infusion of 5-FU and use of cisplatin with lipiodol. In the present study, 24 patients showed disappearance of viable HCC (10 CR patients, 14 PR patients followed by additional therapy). The median survival time of these patients was significantly longer than that of patients with residual HCC. Cisplatin-lipiodol plus 5-FU therapy reduced tumour volume and allowed application of other treatments, thus prolonged survival even in HCC patients with PR. A recent study demonstrated that sorafenib prolongs the survival of patients with advanced HCC.[12] However, the response rate to sorafenib was extremely low.[12] Molecular targeted agents including sorafenib are probably not suitable to make tumour-free.

In the SHARP trial, approximately 52% of patients were reported to have grade 3 or grade 4 treatment-related toxicities, and the most common adverse events were diarrhoea (39%) and hand-foot skin reaction (21%).[12] As HCC is usually accompanied by liver cirrhosis, intensive chemotherapy sometimes induces severe liver damage, leucopaenia and thrombocytopaenia. However, in the present study, adverse events were less frequent than those reported recently in patients who received HAIC, TACE and sorafenib treatment.[12, 17, 37] Only one patient in our cohort developed grade 3 thrombocytopaenia. Furthermore, no deterioration of liver function or evidence of liver damage was noted. Although only two patients showed a transient increase in ascites, they were well controlled by medications. Thus, the adverse effects of this treatment were not serious and controllable.

In conclusion, the present study demonstrated the efficacy of HAIC using cisplatin in lipiodol and 5-FU, and that such therapeutic regimen for unresectable HCC with portal vein tumour thrombosis is not associated with serious adverse effects. The new HAIC can potentially become the first-line treatment for unresectable HCC with portal vein tumour thrombosis, subject to confirmation through a Phase III trial by comparison with sorafenib.

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Production of Infectious Hepatitis C Virus by Using RNA Polymerase I-Mediated Transcription

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In this study, we used an RNA polymerase I (Pol I) transcription system for development of a reverse genetics protocol to produce hepatitis C virus (HCV), which is an uncapped positive-strand RNA virus. Transfection with a plasmid harboring HCV JFH-1 full-length cDNA flanked by a Pol I promoter and Pol I terminator yielded an unspliced RNA with no additional sequences at either end, resulting in efficient RNA replication within the cytoplasm and subsequent production of infectious virions. Using this technology, we developed a simple replicon trans-packaging system, in which transient transfection of two plasmids enables examination of viral genome replication and virion assembly as two separate steps. In addition, we established a stable cell line that constitutively produces HCV with a low mutation frequency of the viral genome. The effects of inhibitors of N-linked glycosylation on HCV production were evaluated using this cell line, and the results suggest that certain step(s), such as virion assembly, intracellular trafficking, and secretion, are potentially up- and downregulated according to modifications of HCV envelope protein glycans. This Pol I-based HCV expression system will be beneficial for a high-throughput antiviral screening and vaccine discovery programs.

Over 170 million people worldwide have been infected with hepatitis C virus (HCV) (22, 33, 37), and persistence of HCV infection is one of the leading causes of liver diseases, such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma (16, 25, 38). The HCV genome is an uncapped 9.6-kb positive-strand RNA sequence consisting of a 5 untranslated region (UTR), an open reading frame encoding at least 10 viral proteins (Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B), and a 3 UTR (46). The structural proteins (Core, E1, and E2) reside in the N-terminal region.

The best available treatment for HCV infection, which is pegylated alpha interferon (IFN-) combined with ribavirin, is effective in only about half of patients and is often difficult to tolerate (25). To date, a prophylactic or therapeutic vaccine is not available. There is an urgent need to develop more effective and better tolerated therapies for HCV infection. Recently, a robust system for HCV production and infection in cultured cells has been developed. The discovery that some HCV isolates can replicate in cell cultures and release infectious particles has allowed the complete viral life cycle to be studied (23, 49, 53). The most robust system for HCV production involves transfection of Huh-7 cells with genomic HCV RNA of the JFH-1 strain by electroporation. However, using this RNA transfection system, the amount of secreted infectious viruses often fluctuate and mutations emerge in HCV genome with multiple passages for an extended period of time (54), which limits its usefulness for antiviral screening and vaccine development.

DNA-based expression systems for HCV replication and virion production have also been examined (5, 15, 21). With DNA-based expression systems, transcriptional expression of functional full-length HCV RNA is controlled by an RNA polymerase II (Pol II) promoter and a self-cleaving ribozyme(s). DNA expression systems using RNA polymerase I (Pol I) have been utilized in reverse genetics approaches to replicate negative-strand RNA viruses, including influenza virus (12, 29), Uukuniemi virus (11), Crimean-Congo hemorrhagic fever virus (10), and Ebola virus (13). Pol I is a cellular enzyme that is abundantly expressed in growing cells and transcribes rRNA lacking both a 5 cap and a 3 poly(A) tail. Thus, viral RNA synthesized in cells transfected with Pol I-driven plasmids containing viral genomic cDNA has no additional sequences at the 5 - or 3 end even in the absence of a ribozyme sequence (28). The advantages of DNA-based expression systems are that DNA expression plasmids are easier to manipulate and generate stable cell lines that constitutively express the viral genome.

We developed here a new HCV expression system based on transfection of an expression plasmid containing a JFH-1 cDNA clone flanked by Pol I promoter and terminator sequences to generate infectious HCV particles from transfected cells. The technology presented here has strong potential to be the basis for trans-encapsidation system by transient transfection of two plasmids and for the establishment of an efficient and reliable screening system for potential antivirals.

MATERIALS AND METHODS

DNA construction. To generate HCV-expressing plasmids containing full-length JFH1 cDNA embedded between Pol I promoter and terminator se-

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quences, part of the 5 UTR region and part of the NS5B to the 3 UTR region of full-length JFH-1 cDNA were amplified by PCR using primers containing BsmBI sites. Each amplification product was then cloned into a pGEM-T Easy vector (Promega, Madison, WI) and verified by DNA sequencing. Both fragments were excised by digestion with NotI and BsmBI, after which they were cloned into the BsmBI site of the pHH21 vector (a gift from Yoshihiro Kawaoka, School of Veterinary Medicine, University of Wisconsin-Madison [29]), which contains a human Pol I promoter and a mouse Pol I terminator. The resultant plasmid was digested by AgeI and EcoRV and ligated to JFH-1 cDNA digested by AgeI and EcoRV to produce pHHJFH1. pHHJFH1/GND having a point mutation at the GDD motif in NS5B to abolish RNA-dependent RNA polymerase activity and pHHJFH1/R783A/R785A carrying double Arg-to-Ala substitutions in the cytoplasmic loop of p7 were constructed by oligonucleotide-directed mutagenesis. To generate pHHJFH1/ E carrying in-frame deletions of parts of the E1 and E2 regions (amino acids [aa] 256 to 567), pHHJFH1 was digested with NcoI and AscI, followed by Klenow enzyme treatment and self-ligation. To generate pHH/SGR-Luc carrying the bicistronic subgenomic HCV reporter replicon and its replication-defective mutant, pHH/SGR-Luc/GND, AgeI-SpeI fragments of pHHJFH1 and pHHJFH1/GND were replaced with an AgeI-SpeI fragment of pSGR-JFH1/Luc (20). In order to construct pCAG/C-NS2 and pCAG/C-p7, PCR-amplified cDNA for C-NS2 and C-p7 regions of the JFH-1 strain were inserted into the EcoRI sites of pCAGGS (30). In order to construct stable cell lines, a DNA fragment containing a Zeocin resistance gene excised from pSV2/Zeo2 (Invitrogen, Carlsbad, CA) was inserted into pHH21 (pHHZeo). Full-length JFH-1 cDNA was then inserted into the BsmBI sites of pHHZeo. The resultant construct was designated pHHJFH1/Zeo.

Cells and compounds. The human hepatoma cell line, Huh-7, and its derivative cell line, Huh7.5.1 (a gift from Francis V. Chisari, The Scripps Research Institute), were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with nonessential amino acids, 100 U of penicillin/ml, 100 g of streptomycin/ml, and 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator. N-Nonyl-deoxynojirimycin (NN-DNJ) and kifunensine (KIF) were purchased from Toronto Research Chemicals (Ontario, Canada), castanospermine (CST) and 1,4-dideoxy-1,4-imino-D-mannitol hydrochloride (DIM) were from Sigma-Aldrich (St. Louis, MO), 1-deoxymannojirimycin (DMJ) and swainsonine (SWN) were from Alexis Corp. (Lausen, Switzerland), and N-butyl-deoxynojirimycin (NB-DNJ) was purchased from Wako Chemicals (Osaka, Japan). BILN 2061 was a gift from Boehringer Ingelheim (Canada), Ltd. These compounds were dissolved in dimethyl sulfoxide and used for the experiments. IFN- was purchased from Dainippon-Sumitomo (Osaka, Japan).

DNA transfection and selection of stable cell lines. DNA transfection was performed by using FuGENE 6 transfection reagent (Roche, Mannheim, German) in accordance with the manufacturer's instructions. To establish stable cell lines constitutively producing HCV particles, pHHJFH1/Zeo was transfected into Huh7.5.1 cells within 35-mm dishes. At 24 h posttransfection (p.t.), the cells were then divided into 100-mm dishes at various cell densities and incubated with DMEM containing 0.4 mg of zeocin/ml for approximately 3 weeks. Selected cell colonies were picked up and amplified. The expression of HCV proteins was confirmed by measuring secreted core proteins. The stable cell line established was designated H751JFH1/Zeo.

In vitro synthesis of HCV RNA and RNA transfection. RNA synthesis and transfection were performed as previously described (26, 49).

RNA preparation, Northern blotting, and RNase protection assay (RPA). Total cellular RNA was extracted with a TRIzol reagent (Invitrogen), and HCV RNA was isolated from filtered culture supernatant by using the QIAamp viral RNA minikit (Qiagen, Valencia, CA). Extracted cellular RNA was treated with DNase (TURBO DNase; Ambion, Austin, TX) and cleaned up by using an RNeasy minikit, which includes another step of RNase-free DNase digestion (Qiagen). The cellular RNA (4 g) was separated on 1% agarose gels containing formaldehyde and transferred to a positively charged nylon membrane (GE Healthcare, Piscataway, NJ). After drying and cross-linking by UV irradiation, hybridization was performed with [-^{32}P]dCTP-labeled DNA using Rapid-Hyb buffer (GE Healthcare). The DNA probe was synthesized from full-length JFH-1 cDNA using the Megaprime DNA labeling system (GE Healthcare). Quantification of positive- and negative-strand HCV RNA was performed using the RPA with biotin-16-uridine-5 -triphosphate (UTP)-labeled HCV-specific RNA probes, which contain 265 nucleotides (nt) complementary to the positive-strand ( ) 5 UTR and 248 nt complementary to the negative-strand ( ) 3 UTR. Human -actin RNA probes labeled with biotin-16-UTP were used as a control to normalize the amount of total RNA in each sample. The RPA was carried out using an RPA III kit (Ambion) according to the manufacturer's procedures. Briefly, 15 g of total cellular RNA was used for hybridization with 0.3 ng of the -actin probe and 0.6 ng of either the HCV ( ) 5 UTR or ( ) 3 UTR RNA probe. After digestion with RNase A/T1, the RNA products were analyzed by electrophoresis in a 6% polyacrylamide–8 M urea gel and visualized by using a chemiluminescent nucleic acid detection module (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions.

Reverse transcriptase PCR (RT-PCR), sequencing, and rapid amplification of cDNA ends (RACE). Aliquots (5 l) of RNA solution extracted from filtered culture supernatant were subjected to reverse transcription with random hexamer and Superscript II reverse transcriptase (Invitrogen). Four fragments of HCV cDNA (nt 129 to 2367, nt 2285 to 4665, nt 4574 to 7002, and nt 6949 to 9634), which covers most of the HCV genome, were amplified by nested PCR. Portions (1 or 2 l) of each cDNA sample were subjected to PCR with TaKaRa LA Taq polymerase (Takara, Shiga, Japan). The PCR conditions consisted of an initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 3 min. The amplified products were separated by agarose gel electrophoresis and used for direct DNA sequencing. To establish the 5 ends of the HCV transcripts from pHHJFH1, a synthetic 45-nt RNA adapter (Table 1) was ligated to RNA extracted from the transfected cells 1 day p.t. using T4 RNA ligase (Takara). The viral RNA sequences were then reverse transcribed using SuperScript III reverse transcriptase (Invitrogen) with a primer, RT (Table 1). The resultant cDNA sequences were subsequently amplified by PCR with 5 RACEouter-S and 5 RACEouter-R primers, followed by a second cycle of PCR using 5 RACEinner-S and 5 RACEinner-R primers (Table 1). To establish the terminal 3 -end sequences, extracted RNA sequences were polyadenylated using a poly(A) polymerase (Takara), reverse transcribed with CAC-T35 primer (Table 1), and amplified with the primers 3X-10S (Table 1) and CAC-T35. The amplified 5 and 3 cDNA sequences were then separated by agarose gel electrophoresis, cloned into the pGEM-T Easy vector (Promega), and sequenced.

Western blotting. The proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA) after separation by SDS-PAGE. After blocking, the membranes were probed with a mouse monoclonal anti-HCV core antibody (2H9) (49), a rabbit polyclonal anti-NS5B antibody, or a mouse monoclonal GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (Chemicon, Temecula, CA), followed by incubation with a peroxidase-conjugated secondary antibody and visualization with an ECL Plus Western blotting detection system (Amersham, Buckinghamshire, United Kingdom).

Quantification of HCV core protein. HCV core protein was quantified by using a highly sensitive enzyme immunoassay (Ortho HCV antigen ELISA kit; Ortho Clinical Diagnostics, Tokyo, Japan) in accordance with the manufacturer's instructions.

Sucrose density gradient analysis. Samples of cell culture supernatant were processed by low-speed centrifugation and passage through a 0.45- m-pore-size filter. The filtrated supernatant was then concentrated 30-fold by ultrafiltration by using an Amicon Ultra-15 filter device with a cutoff molecular mass of 100,000 kDa (Millipore), after which it was layered on top of a continuous 10 to 60% (wt/vol) sucrose gradient, followed by centrifugation at 35,000 rpm at 4°C for 14 h with an SW41 rotor (Beckman Coulter, Fullerton, CA). Fractions of 1 ml were collected from the bottom of the gradient. The core level and infectivity of HCV in each fraction were determined.

Quantification of HCV infectivity. Infectious virus titration was performed by a 50% tissue culture infectious dose ($TCID_{50}$) assay, as previously described (23, 26). Briefly, naive Huh7.5.1 cells were seeded at a density of 10^4 cells/well in a 96-well flat-bottom plate 24 h prior to infection. Five serial dilutions were performed, and the samples were used to infect the seeded cells (six wells per dilution). At 72 h after infection, the inoculated cells were fixed and immunostained with a rabbit polyclonal anti-NS5A antibody (14), followed by an Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Invitrogen).

Labeling of de novo-synthesized viral RNA and immunofluorescence staining. Labeling of de novo-synthesized viral RNA was performed as previously described with some modifications (40). Briefly, cells were plated onto an eight-well chamber slide at a density of 5 10^4 cells/well. One day later, the cells were incubated with actinomycin D at a final concentration of 10 g/ml for 1 h and washed twice with HEPES-saline buffer. Bromouridine triphosphate (BrUTP) at 2 mM was subsequently transfected into the cells using FuGENE 6 transfection reagent, after which the cells were incubated for 15 min on ice. After the cells were washed twice with phosphate-buffered saline (PBS), they were incubated in fresh DMEM supplemented with 10% FBS at 37°C for 4 h. The cells were then fixed with 4% paraformaldehyde for 20 min and permeabilized with PBS containing 0.1% Triton X-100 for 15 min at room temperature. Immunofluorescence staining of NS5A and de novo-synthesized HCV RNA was performed as previously described (26, 40). The nuclei were stained with DAPI (4 ,6 -diamidino-2-phenylindole) solution (Sigma-Aldrich). Confocal microscopy was performed

TABLE 1. Oligonucleotides used for RT-PCR and RACE of the JFH-1 genome

Method or segment	Oligonucleotide	Sequences (5 –3)
5 RACE	RT	GTACCCCATGAGGTCGGCAAAG
	45-nt RNA adapter	GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA
	5 RACEouter-S	GCTGATGGCGATGAATGAACACTG
	5 RACEouter-R	GACCGCTCCGAAGTTTTCCTTG
	5 RACEinner-S	GAACACTGCGTTTGCTGGCTTTGATG
	5 RACEinner-R	CGCCCTATCAGGCAGTACCACAAG
3 RACE	CAC-T35	CACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
	3X-10S	ATCTTAGCCCTAGTCACGGC
nt 129-2367	44S (1st PCR)	CTGTGAGGAACTACTGTCTT
	2445R	TCCACGATGTTCTGGTGAAG
	17S (2nd PCR)	CGGGAGAGCCATAGTGG
	2367R	CATTCCGTGGTAGAGTGCA
nt 2285-4665	2099S (1st PCR)	ACGGACTGTTTTAGGAAGCA
	4706R	TTGCAGTCGATCACGGAGTC
	2285S (2nd PCR)	AACTTCACTCGTGGGGATCG
	4665R	TCGGTGGCGACGACCAC
nt 4574-7002	4547S (1st PCR)	AAGTGTGACGAGCTCGCGG
	7027R	CATGAACAGGTTGGCATCCACCAT
	4594S (2nd PCR)	CGGGGTATGGGCTTGAACGC
	7003R	GTGGTGCAGGTGGCTCGCA
nt 6949-9634	6881S (1st PCR)	ATTGATGTCCATGCTAACAG
	3X-75R	TACGGCACTCTCTGCAGTCA
	6950S (2nd PCR)	GAGCTCCTCAGTGAGCCAG
	3X-54R	GCGGCTCACGGACCTTTCAC

using a Zeiss confocal laser scanning microscope LSM 510 (Carl Zeiss, Oberkochen, Germany).

Luciferase assay. Huh7.5.1 cells were seeded onto a 24-well cell culture plate at a density of 3 10^4 cells/well 24 h prior to inoculation with 100 l of supernatant from the transfected cells. The cells were incubated for 72 h, followed by lysis with 100 l of lysis buffer. The luciferase activity of the cells was determined by using a luciferase assay system (Promega). All luciferase assays were done at least in triplicate. For the neutralization experiments, a mouse monoclonal anti-CD81 antibody (JS-81; BD Pharmingen, Franklin Lakes, NJ) and a mouse monoclonal anti-FLAG antibody (Sigma-Aldrich) were used.

Flow cytometric analysis. Cells detached by treatment with trypsin were incubated in PBS containing 1% (vol/vol) formaldehyde for 15 min. A total of 5 10^5 cells were resuspended in PBS and treated with or without 0.75 g of anti-CD81 antibody for 30 min at 4°C. After being washed with PBS, the cells were incubated with an Alexa Fluor 488-conjugated anti-mouse secondary antibody (Invitrogen) at 1:200 for 30 min at 4°C, washed repeatedly, and resuspended in PBS. Analyses were performed by using FACSCalibur system (Becton Dickinson, Franklin Lakes, NJ).

RESULTS

Analysis of the 5 and 3 ends of HCV RNA sequences generated from Pol I-driven plasmids. To examine whether the HCV transcripts generated from Pol I-driven plasmids had correct nucleotides at the 5 and 3 ends, we extracted RNA from Huh-7 cells transfected with pHHJFH1, which carries a genome-length HCV cDNA with a Pol I promoter/terminator, as well as from the culture supernatants. After this, the nucleotide sequences at both ends were determined using RACE and sequence analysis. A 328-nt fragment corresponding to cDNA from the 5 end of HCV RNA was detected in the cell samples (Fig. 1A). Cloning of amplified fragments confirmed that the HCV transcripts were initiated from the first position of the viral genome in all of the clones sequenced (Fig. 1B). Similarly, a 127-nt amplification fragment was detected in each sample by 3 RACE (Fig. 1C), and the same 3 -end nucleotide sequence was observed in all clones derived from the culture supernatant (Fig. 1D, left). An additional two nucleotides (CC) were found at the 3 end of the HCV transcript in a limited number of sequences (1 of 11 clones) derived from the cell sample (Fig. 1D, right), which were possibly derived from the Pol I terminator sequence by incorrect termination. These results indicate that most HCV transcripts generated from the Pol I-based HCV cDNA expression system are faithfully processed, although it is not determined whether the 5 terminus of the viral RNA generated from Pol I system is triphosphate or monophosphate. It can be speculated that viral RNA lacking modifications at the 5 and 3 ends is preferentially packaged and secreted into the culture supernatant.

Production of HCV RNA, proteins, and virions from cells transiently transfected with Pol I-driven plasmids. To examine HCV RNA replication and protein expression in cells transfected with pHHJFH1, pHHJFH1/GND, or virion production-defective mutants, pHHJFH1/ E and pHHJFH1/R783A/R785A, which possess an in-frame deletion of E1/E2 region and substitutions in the p7 region, respectively (19, 42, 49), RPA and Western blotting were performed 5 days p.t. (Fig. 2A, B, and D). Positive-strand HCV RNA sequences were more abundant than negative-strand RNA sequences in these cells. Positive-strand RNA, but not negative-strand RNA, was detected in cells transfected with the replication-defective mutant pHHJFH1/GND (Fig. 2A and B). Northern blotting showed that genome-length RNA was generated in pHHJFH1-transfected cells but not in pHHJFH1/GND-transfected cells (Fig. 2C).

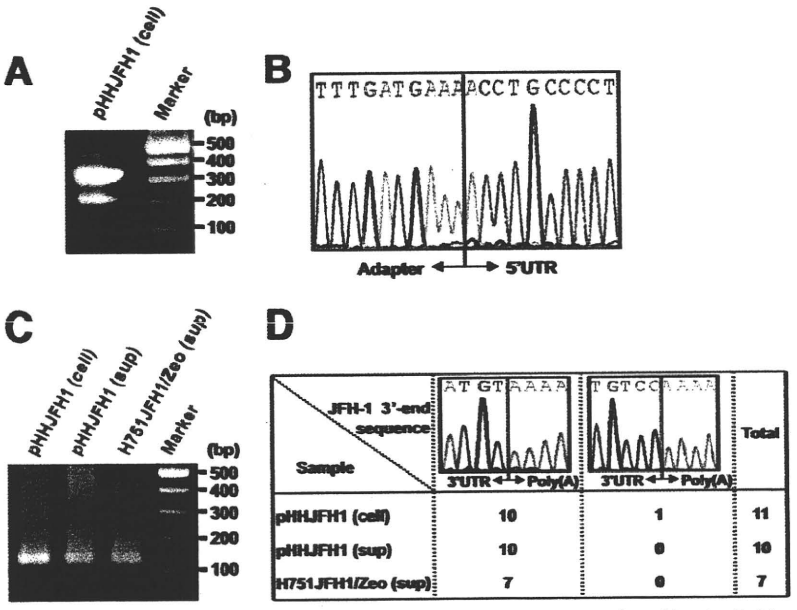

FIG. 1. Determination of the nucleotide sequences at the 5 -and 3 ends of HCV RNA produced by the Pol I system. (A and B) 5 RACE and sequence analysis. A synthesized RNA adapter was ligated to RNA extracted from cells transfected with pHHJFH1. The positive-strand HCV RNA was reverse transcribed, and the resulting cDNA was amplified by nested PCR. The amplified 5 -end cDNA was separated by agarose gel electrophoresis (A), cloned, and sequenced (B). (C and D) 3 RACE and sequence analysis. RNA extracted from pHHJFH1-transfected cells, the culture supernatant of transfected cells, and the culture supernatant of H751JFH1/Zeo cells were polyadenylated, reverse transcribed, and amplified by PCR. The amplified 3 -end cDNA was separated by agarose gel electrophoresis (C), cloned, and sequenced (D).

As shown in Fig. 2D, the intracellular expression of core and NS5B proteins was comparable among cells transfected with pHHJFH1, pHHJFH1/ E, and pHHJFH1/R783A/R785A. Neither viral protein was detected in pHHJFH1/GND-transfected cells, suggesting that the level of viral RNA generated transiently from the DNA plasmid does not produce enough HCV proteins for detection and that ongoing amplification of the HCV RNA by the HCV NS5B polymerase allows a high enough level of viral RNA to produce detectable levels of HCV proteins.

To assess the release of HCV particles from cells transfected with Pol I-driven plasmids, core protein was quantified in culture supernatant by enzyme-linked immunosorbent assay (ELISA) or sucrose density gradient centrifugation. Core protein secreted from pHHJFH1-transfected cells was first detectable 2 days p.t., with levels increasing up to 4 pmol/liter on day 6 (Fig. 3A). This core protein level was 4- to 6-fold higher than that in the culture supernatant of pHHJFH1/ E- or pHHJFH1/R783A/R785A-transfected cells, despite comparable intracellular core protein levels (Fig. 2D). Core protein was not secreted from cells transfected with pHHJFH1/GND (Fig. 3A). In another experiment, a plasmid expressing the secreted form of human placental alkaline phosphatase (SEAP) was cotransfected with each Pol I-driven plasmid. SEAP activity in culture supernatant was similar among all transfection groups, indicating comparable efficiencies of transfection (data not shown). Sucrose density gradient analysis of the concentrated supernatant of pHHJFH1-transfected cells indicated that the distribution of core protein levels peaked in the fraction of 1.17 g/ml density, while the peak of infectious titer was observed in the fraction of 1.12 g/ml density (Fig. 3B), which is consistent with the results of previous studies based on JFH-1-RNA transfection (23).

We next compared the kinetics of HCV particle secretion in the Pol I-driven system and RNA transfection system. Huh-7 cells, which have limited permissiveness for HCV infection (2), were transfected with either pHHJFH1 or JFH-1 RNA, and then cultured by passaging every 2 or 3 days. As shown in Fig. 3C, both methods of transfection demonstrated similar kinetics of core protein levels until 9 days p.t., after which levels gradually fell. However, significantly greater levels of core protein were detected in the culture of pHHJFH1-transfected cells compared to the RNA-transfected cells on day 12 and 15 p.t. This is likely due to an ongoing production of positive-strand viral RNA from transfected plasmids since RNA degradation generally occurs more quickly than that of circular DNA.

Establishment of stable cell lines constitutively producing HCV virion. To establish cell lines with constitutive HCV production, pHHJFH1/Zeo carrying HCV genomic cDNA and the Zeocin resistance gene were transfected into Huh7.5.1 cells. After approximately 3 weeks of culture with zeocin at a concentration of 0.4 mg/ml, cell colonies producing HCV core protein were screened by ELISA, and three clones were identified that constitutively produced the viral protein (H751JFH1/Zeo cells). Core protein levels within the culture supernatant of selected clones (H751-1, H751-6, and H751-50) were 2.0 10^4, 2.7 10^3, and 1.4 10^3 fmol/liter, respectively. Clone H751-1 was further analyzed. Indirect immunofluorescence with an anti-NS5A antibody showed fluorescent staining of NS5A in the cytoplasm of almost all H751JFH1/

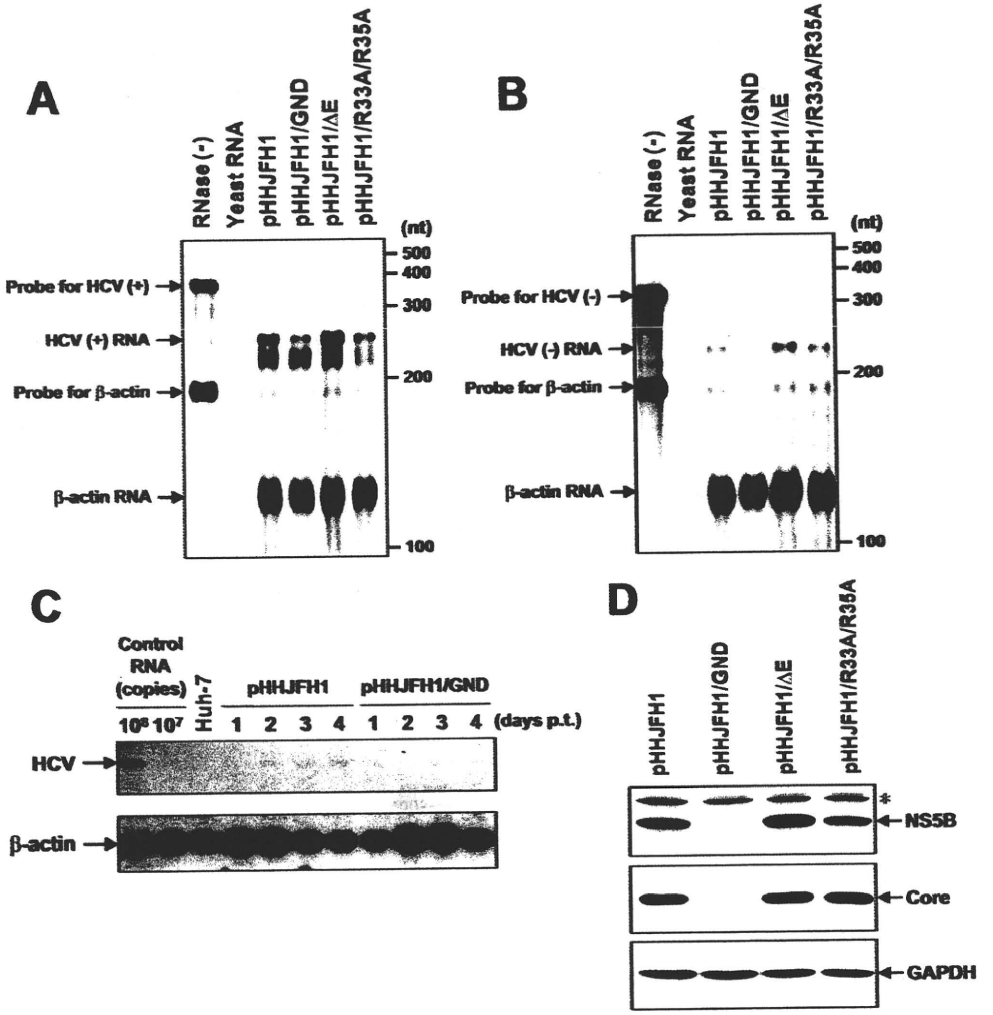

FIG. 2. HCV RNA replication and protein expression in cells transfected with Pol I-driven plasmids. (A and B) Assessment of HCV RNA replication by RPA. Pol I-driven HCV-expression plasmids were transfected into Huh-7 cells. Total RNA was extracted from the cells on day 5 p.t. and positive (A)- and negative (B)-strand HCV RNA levels were determined by RPA as described in Materials and Methods. In the RNase ( ) lanes, yeast RNA mixed with RNA probes for HCV and human -actin were loaded without RNase A/T1 treatment. In the yeast RNA lanes, yeast RNA mixed with RNA probes for HCV and human -actin were loaded in the presence of RNase A/T1. (C) Northern blotting of total RNAs prepared from the transfected cells. Huh-7 cells transfected with pHHJFH1 or pHHJFH1/GND were harvested for RNA extraction through days 1 to 4 p.t. Control RNA, given numbers of synthetic HCV RNA; Huh-7, RNA extracted from naive cells. Arrows indicate full-length HCV RNA and -actin RNA. (D) HCV protein expression in the transfected cells. Pol I-driven HCV-expression plasmids were transfected into Huh-7 cells, harvested, and lysed on day 6 p.t. The expression of NS5B, core, and GAPDH was analyzed by Western blotting as described in Materials and Methods. The asterisk indicates nonspecific bands.

Zeo cells (Fig. 4A), whereas no signal was detected in parental Huh7.5.1 cells (Fig. 4B). To determine where HCV RNA replicates in H751JFH1/Zeo cells, labeling of de novo-synthesized HCV RNA was performed. After interfering with mRNA production by exposure to actinomycin D, BrUTP-incorporated de novo-synthesized HCV RNA was detected in the cytoplasm of H751JFH1/Zeo cells (Fig. 4D) colocalized with NS5A in the perinuclear area (Fig. 4E and F).

Low mutation frequency of the viral genome in a long-term culture of H751JFH1/Zeo cells. The production level of infectious HCV from H751JFH1/Zeo cells at a concentration of 10^3 $TCID_{50}$/ml was maintained over 1 year of culture (data not shown). It has been shown that both virus and host cells may adapt during persistent HCV infection in cell cultures, such that cells become resistant to infection due to reduced expression of the viral coreceptor CD81 (54). As shown in Fig. 5, we analyzed the cell surface expression of CD81 on the established cell lines by flow cytometry and observed markedly reduced expression on H751JFH1/Zeo cells compared to parental Huh7.5.1 cells. It is therefore possible that only a small proportion of HCV particles generated from H751JFH1/Zeo cells enter and propagate within the cells. The H751JFH1/Zeo system is thought to result in virtually a single cycle of HCV production from the chromosomally integrated gene and thus

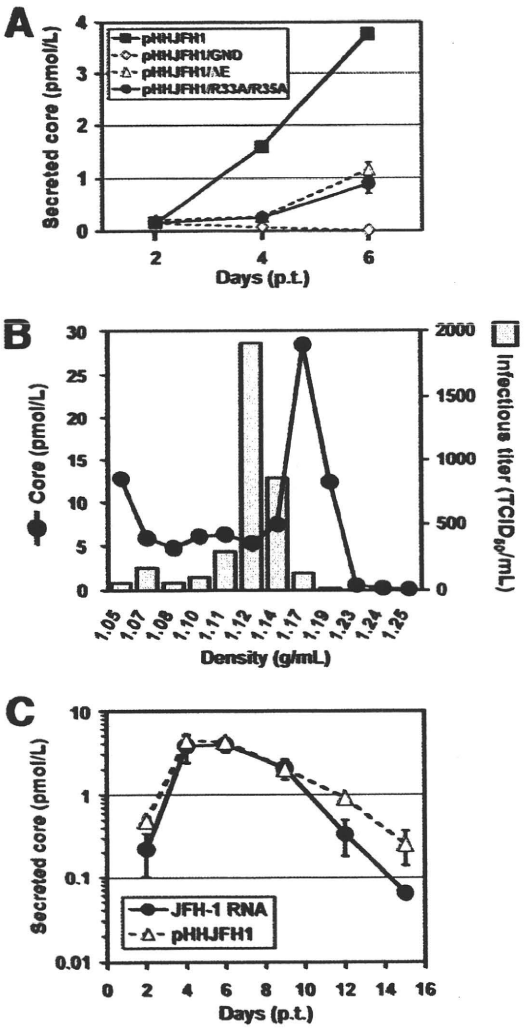

FIG. 3. HCV released from cells transfected with Pol I-driven plasmids. (A) HCV particle secretion from the transfected cells. The culture supernatant of Huh-7 cells transfected with Pol I-driven plasmids containing wild-type or mutated HCV genome were harvested on days 2, 4, and 6 and assayed for HCV core protein levels. The data for each experiment are averages of triplicate values with error bars showing standard deviations. (B) Sucrose density gradient analysis of the culture supernatant of pHHJFH1-transfected cells. Culture supernatant collected on day 5 p.t. was cleared by low-speed centrifugation, passed through a 0.45- m-pore-size filter, and concentrated 30-fold by ultrafiltration. After fractionating by sucrose density gradient centrifugation, the core protein level and viral infectious titer of each fraction were measured. (C) Kinetics of core protein secretion from cells transfected with pHHJFH1 or with JFH-1 genomic RNA. A total of 10^6 Huh-7 cells were transfected with 3 g of pHHJFH1 or the same amount of in vitro-transcribed JFH-1 RNA by electroporation. The cells were passaged every 2 to 3 days before reaching confluence. Culture supernatant collected on the indicated days was used for core protein measurement. The level of secreted core protein (pmol/liter) is expressed on a logarithmic scale. The data for each experiment are averages of triplicate values with error bars showing standard deviations.

may yield a virus population with low mutation frequencies. To further examine this, we compared HCV genome mutation rates following production from H751JFH1/Zeo cells compared to cells constitutively infected with HCV after serial passages. RNAs were extracted from the supernatant of H751JFH1/Zeo cells cultured for 120 days, and cDNA sequences were amplified by nested PCR with four sets of primers encompassing almost the entire HCV genome (Table 1). PCR products with expected sizes of 2 to 2.5 kb were obtained [Fig. 6A, RT( )] and subjected to direct sequencing. No amplified product was detected in samples without reverse transcription [Fig. 6A, RT( )], suggesting no DNA contamination in culture supernatants or extracted RNA solutions. As shown in Fig. 5B (upper panel), three nucleotide mutations, including two substitutions in the E1 (nt 1218) and E2 (nt 1581) regions, and one deletion in the 3 UTR (nt 9525) were found within the HCV genome with the mutation rate calculated at 9.6 10^{-4} base substitutions/site/year. These mutations were not detected in the chromosomally integrated HCV cDNA (data not shown). The present results also indicate that no splicing of the viral RNA occurred in the Pol I-based HCV JFH-1 expression system. The HCV genome sequence produced by JFH-1 virus-infected Huh7.5.1 cells was analyzed in the same way using culture supernatant 36 days after RNA transfection. As shown in Fig. 6B (lower panel), 10 mutations, including five substitutions throughout the open reading frame and five deletions in the 3 UTR, were detected, and the mutation rate was calculated at 1.1 10^{-2} base substitutions/site/year.

Effects of glycosylation inhibitors on HCV production. It is known that N-linked glycosylation and oligosaccharide trimming of a variety of viral envelope proteins including HCV E1 and E2 play key roles in the viral maturation and virion production. To evaluate the usefulness of the established cell line for antiviral testing, we determined the effects of glycosylation inhibitors, which have little to no cytotoxicity at the concentrations used, on HCV production in a three day assay using H751JFH1/Zeo cells. The compounds tested are known to inhibit the endoplasmic reticulum (ER), Golgi-resident glucosidases, or mannosidases that trim glucose or mannose residues from N-linked glycans. Some are reported to be involved in proteasome-dependent or -independent degradation of misfolded or unassembled glycoproteins to maintain protein integrity (4, 8, 27, 35).

As shown in Fig. 7A and B, treatment of H751JFH1/Zeo cells with increasing concentrations of NN-DNJ, which is an inhibitor of ER -glucosidases, resulted in a dose-dependent reduction in secreted core protein. NN-DNJ was observed to have an IC_{50} (i.e., the concentration inhibiting 50% of core protein secretion) of 20 M. In contrast, KIF, which is an ER -mannosidase inhibitor, resulted in a 1.5- to 2-fold increase in secreted core protein compared to control levels. The other five compounds did not significantly change core protein levels. We further determined the effects of NN-DNJ and KIF on the production of infectious HCV (Fig. 7C). As expected, NN-DNJ reduced the production of infectious virus in a dose-dependent manner, while production increased in the presence of KIF at 10 to 100 M. Since NN-DNJ and KIF did not significantly influence viral RNA replication, as determined using the subgenomic replicon (data not shown), the present results suggest that some step(s), such as virion assembly, intracellular trafficking, and secretion, may be up- or downregulated depending on glycan modifications of HCV envelope proteins within the ER. Inhibitory effect of NN-DNJ was reproducibly observed using the cell line after 1 year of culturing

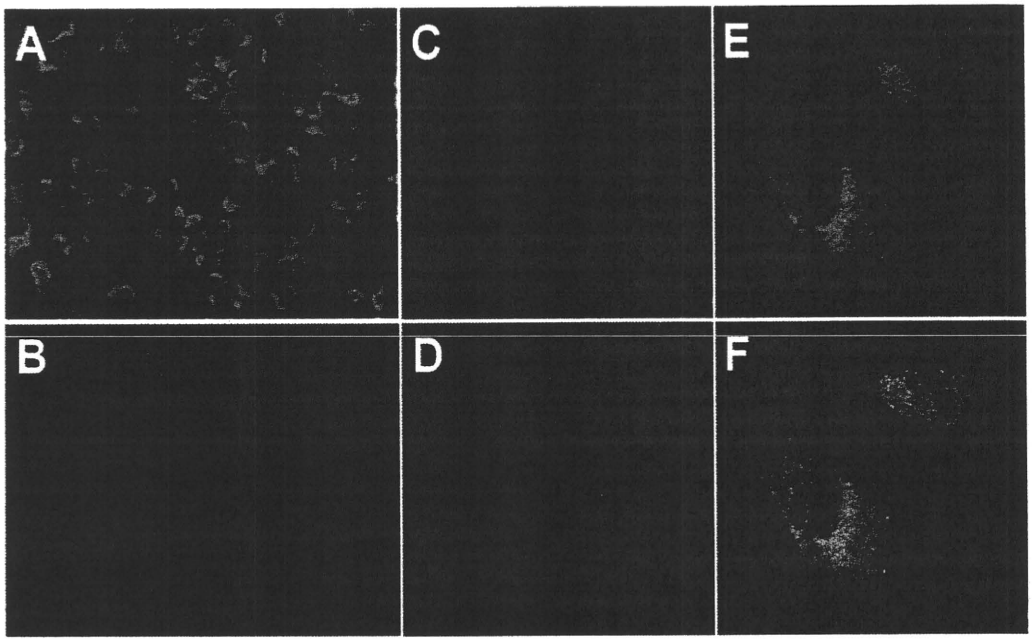

FIG. 4. Indirect immunofluorescence analysis of H751JFH1/Zeo cells. (A and B) H751JFH1/Zeo cells (A) and parental Huh7.5.1 cells (B) were immunostained with an anti-NS5A antibody. (C to F) The subcellular colocalization of de novo-synthesized HCV RNA and NS5A in H751JFH1/Zeo cells was analyzed. The cells were stained with DAPI (C), an anti-bromodeoxyuridine antibody (D), and an anti-NS5A antibody (E). The merge panel is shown in panel F.

(Fig. 7D). Under the same condition, the core protein secretion was inhibited by 28 and 58% with 10 and 100 nM BILN 2061, an NS3 protease inhibitor, respectively (Fig. 7D).

Replicon trans-packaging system. Recently, ourselves and others have developed a packaging system for HCV subgenomic replicon RNA sequences by providing trans viral core-NS2 proteins (1, 17, 41). Since viral structural proteins are not encoded by the subgenomic replicon, progeny virus cannot be produced after transfection. Thus, the single-round infectious HCV-like particle (HCV-LP) generated by this system potentially improves the safety of viral transduction. Here, in order to make the trans-packaging system easier to manipulate, we used a Pol I-driven plasmid to develop a transient two-plasmid expression system for the production of HCV-LP. pHH/SGR-Luc, which carries a bicistronic subgenomic reporter replicon with a Pol I promoter/terminator, or its replication-defective mutant, were cotransfected with or without a core-NS2 expression plasmid (Fig. 8A). The culture supernatant was then collected between days 2 and 5 p.t. and used to inoculate naive Huh7.5.1 cells. Reporter luciferase activity, as a quantitative measure of infectious virus production, was assessed in the cells 3 days postinoculation. As shown in Fig. 8B, reporter replication activity was easily detectable in cells inoculated with culture supernatant from cells cotransfected with pHH/

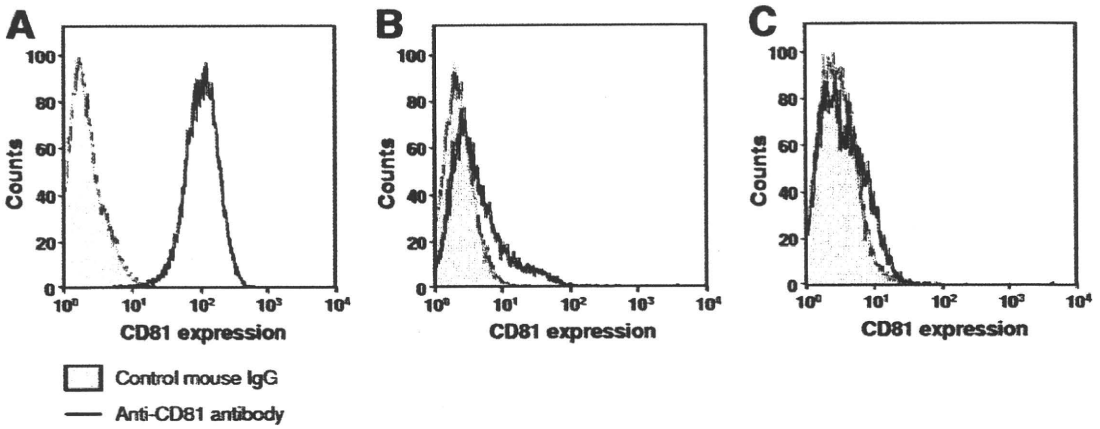

FIG. 5. Loss of CD81 expression in H751JFH1/Zeo cells. The cell surface expression of CD81 on Huh7.5.1 cells (A), H751JFH1/Zeo clone H751-1 (B), and clone H751-50 (C) was analyzed by flow cytometry after being stained with anti-CD81 antibody.