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The genome of herpesvirus saimiri C488 which is capable of transforming human T cells

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Abstract

Herpesvirus saimiri (HVS), the rhadinovirus prototype, is apathogenic in the persistently infected natural host, the squirrel monkey, but causes acute T cell leukemia in other New World primate species. In contrast to subgroups A and B, only strains of HVS subgroup C such as C488 are capable of transforming primary human T cells to stable antigen-independent growth in culture. Here, we report the complete 155-kb genome sequence of the transformation-competent HVS strain C488. The A+T-rich unique L-DNA of 113,027 bp encodes at least 77 open reading frames and 5 URNAs. In addition to the viral oncogenes *stp* and *tip*, only a few genes including the transactivator *orf50* and the glycoprotein *orf51* are highly divergent. In a series of new primary HVS isolates, the subgroup-specific divergence of the *orf50/orf51* alleles was studied. In these new isolates, the *orf50/orf51* alleles of the respective subgroup segregate with the *stp* and/or *tip* oncogene alleles, which are essential for transformation.

Keywords: Growth transformation; Herpesvirus; Leukemia; Lymphoma; Rhadinovirus; *Saimiri sciureus;* Squirrel monkey; T-lymphocyte; Tumor virus

Introduction

Herpesvirus saimiri (HVS) or saimiriine herpesvirus 2 (SaHV-2) was isolated initially from a kidney cell culture of a healthy squirrel monkey (*Saimiri sciureus*) (Melendez et al., 1968). Although HVS does not cause disease in its natural host, it induces malignant lymphoma after experimental infection of other New World primate species and rabbits (Daniel et al., 1974; Melendez et al., 1969; reviewed in Fickenscher and Fleckenstein, 2001; Fleckenstein and Desrosiers, 1982). Squirrel monkeys are an endangered species of South American rain forests, but are common in zoos and primate research facilities. Most squirrel monkeys become seropositive during the first year of age and are then persistently infected by HVS. However, there are no reports of human tumors associated with squirrel monkeys or HVS.

HVS belongs to the subfamily *Gammaherpesvirinae.* In contrast to Epstein-Barr virus (EBV, HHV-4) of the genus *Lymphocryptovirus* (γ_1-herpesviruses), HVS was assigned to the genus *Rhadinovirus* (γ_2-herpesviruses). The prototype rhadinovirus, the HVS strain A11 (Albrecht et al., 1992a; Falk et al., 1972), is leukemogenic in New World monkeys such as *Aotus trivirgatus*, *Saguinus oedipus*, and *Callithrix jacchus* and transforms *C. jacchus* T cells in vitro to interleukin (IL)-2-independent growth (Desrosiers et al., 1986; Szomolanyi et al., 1987). However, strain A11 is unable to transform human T cells in culture. The saimiri transformation-associated protein of subgroup A gene (*stpA*) is required for T cell transformation in vitro and leukemogenesis (Desrosiers et al., 1985, 1986; Murthy et al., 1989). The HVS strain A11 was the first sequenced rhadinovirus genome (Albrecht et al., 1992a). A genomic variability between different HVS isolates was detected by restriction fragment mapping (Desrosiers and Falk, 1982) and by cross-hybridization with probes from the terminal coding region (Medveczky et al., 1984).

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The HVS strain C488 from a healthy squirrel monkey of the New England Primate Research Center (Southborough, MA) had a restriction enzyme fragment pattern distinct from strain A11 (Desrosiers and Falk, 1982). After in vitro infection of primary human blood cells with HVS strain C488, a reproducible outgrowth of T cell lines was observed which stably proliferated without restimulation with antigen or mitogen for more than 18 months of continuous culture (Biesinger et al., 1992). The HVS-transformed T cell lines resemble activated mature $CD4^+$ or $CD8^+$ T cells and mostly remain dependent on exogenous IL-2. Remarkably, HVS-transformed human T cells retain the antigen specificity of their parental clones (reviewed in Fickenscher and Fleckenstein, 2001).

Besides C488, a few other subgroup C strains such as C484 and C139 were also reported to transform primary human T cells, however, apparently less efficiently (Chou et al., 1995; Fickenscher et al., 1997; Medveczky et al., 1993). The strain C488 was used most frequently for this application (reviewed in Fickenscher and Fleckenstein, 2002). HVS C488 was pathogenic in *S. oedipus* and *C. jacchus* and transformed T cells from New and Old World primates in vitro to antigen independent growth (Akari et al., 1996; Biesinger et al., 1992; Duboise et al., 1998b; Feldmann et al., 1997; Knappe et al., 1998b, 2000; Meinl et al., 1997). Although the reinfusion of autologous ex vivo transformed macaque T cells was well tolerated, HVS C488 induced lymphoproliferative disease after high-dose viral infection in the same species (Alexander et al., 1997; Knappe et al., 2000).

The genes *stpC* and *tip* of HVS C488 are both required for viral transformation and pathogenicity (Duboise et al., 1998b; Knappe et al., 1997). Both genes are expressed from a common bicistronic mRNA which is transcriptionally induced by T cell activation (Fickenscher et al., 1996). *stpC* and *stpA* were oncogenic when expressed in rodent fibroblasts or transgenic animals (Jung et al., 1991; Kretschmer et al., 1996; Murphy et al., 1994). StpC was shown to bind cellular Ras, to activate mitogen-activated protein kinase, and to activate the NFκB pathway through interaction with tumor necrosis factor receptor-associated factors (TRAF) (Jung and Desrosiers, 1995; Lee et al., 1999). StpC multimerization via its collagen-like domain is important for TRAF association (Choi et al., 2000). StpC mutants with impaired TRAF binding were nevertheless capable of transforming marmoset lymphocytes, but were deficient for the transformation of human T cells and rodent fibroblasts in vitro (Lee et al., 1999). In contrast to StpC, the TRAF interaction of StpA and StpB does not activate NFκB (Choi et al., 2000; Lee et al., 1999), though StpA is able to transform rodent fibroblasts (Jung et al., 1991). StpA and StpB bind to the Src-homology 2 domain of Src which phosphorylates StpB in vitro and in vivo (Choi et al., 2000; Hör et al., 2001).

The first open reading frame (ORF) in the unique L-DNA of HVS C488 and other subgroup C strains encodes a protein that binds to the tyrosine kinase Lck in transformed T cells, termed tyrosine kinase-interacting protein or Tip (Biesinger et al., 1990, 1995; Fickenscher et al., 1997; Lund et al., 1995, 1996). Although *tip* does not exert transforming activity in fibroblasts, it induces T cell lymphoma in conditionally transgenic mice (Wehner et al., 2001). Thus, *tip* and *stpC* are the oncogenes of HVS subgroup C strains. Tip associates with Lck via the proline-rich Src-homology region 3 binding (SH3B) and the C-terminal Src-kinase homology domain (CSKH) (Jung et al., 1995a). In many test systems, the Tip-interaction resulted in enzymatic Lck activation (Fickenscher et al., 1997; Hartley et al., 1999; Lund et al., 1997a; Noraz et al., 1998; Wiese et al., 1996). Both the SH3B and the CSKH domains of Tip were required for Lck activation (Hartley et al., 2000). However, when stably expressed in transfected cell lines, Tip caused a marked inhibition of Lck-mediated signal transduction (Jung et al., 1995b). Surprisingly, the mutation of the Tip SH3B domain did not block marmoset T cell transformation (Duboise et al., 1998c). Moreover, the Tip mutant Y114S further enhanced Lck downregulation and reversed the transformed phenotype of cells with a constitutively active Lck mutant (Guo et al., 1997). The phosphorylation of exactly this tyrosine residue in Tip is required for the binding of signal transducer and activator of transcription (STAT) factors (Hartley and Cooper, 2000). Tip induces an Lck-dependent activation of STAT1 and STAT3 and promotes STAT-dependent transcription (Lund et al., 1997b, 1999).

Since the transforming *stp* and *tip* genes are necessary for transformation and pathogenicity (Duboise et al., 1998b; Knappe et al., 1997), they are the major determinants for the transforming potential of the different HVS strains. However, as experiments with retroviral *stp* and *tip* expression constructs in absence of the herpesvirus genome have not been successful, it is not clear if *stp* and *tip* are sufficient for transformation. Therefore, we have now established the entire nucleotide sequence of HVS C488 to learn which additional viral factors are variable and, thus, might contribute to the different phenotypes of the HVS subgroups.

Results and discussion

The genome of HVS C488

The genome length of the packaged linear virion DNA was determined with density gradient-purified DNA by pulse-field electrophoresis. The maximal signal intensity corresponded to an entire genome size of approximately 155 kb, ranging from 130 to 165 kb (Fig. 1). Digestion with the restriction endonuclease *Eag*I yielded a linear fragment of approximately 113 kb covering the coding unique L-DNA, and in addition, two supermolar fragments of 1.3 and 1.45 kb corresponding to the terminal repetitive H-DNA (data

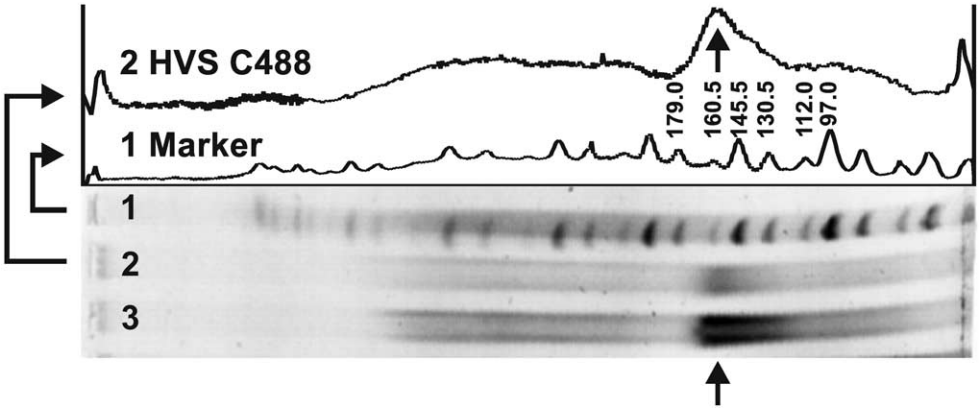

Fig. 1. HVS genome size. Clamped homogenous electric field electrophoresis was performed with purified virion DNA of HVS strain C488 (lanes 2 and 3). Marker (lane 1): mid-range DNA marker (New England Biolabs). Top, a densitometric evaluation of the gel is shown.

not shown). Thus, the H-DNA comprises 40 kb (17 to 52 kb) or on average 25% of the packaged DNA genome.

Various strategies were combined for sequencing of the entire herpesvirus genome: Either cloned *Xba*I or *Hind*III restriction fragments, or cloned random fragments from sonicated DNA, or PCR fragments were sequenced. Larger fragments in plasmids or cosmids were covered by primer walking. Totally, 1757 independent sequence readings yielded 898,756 bp of uncompiled raw data that were assembled into a contiguous sequence of 113,027 bp A+T-rich unique L-DNA and the terminal repetitive G+C-rich H-DNA of 1458 bp. The average redundance was 8.1, and all regions were covered by at least two readings on each of both strands. The terminal G+C-rich H-DNA repeats of strain C488 are composed of two units of different sizes. The *Eag*I restriction enzyme recognition site defines two distinct repeat units of 1318 and 1458 bp. The shorter unit represents the longer repeat unit of which 140 bp are deleted. The deleted area includes the putative cleavage signals of the virus packaging enzymes (Bankier et al., 1985; Stamminger et al., 1987). The H- to L-DNA transition region contains a stretch of rearranged G+C-rich DNA which cannot be perfectly aligned to the H-DNA repeat sequences (Albrecht et al., 1992a). For the C488 L-DNA sequence, we defined the H-/L-DNA transition site and the left genomic terminus of the L-DNA as the first predicted packaging cleavage site of the H-DNA. Accordingly, the L-DNA between positions 1 to 622 is G+C-rich and consists of rearranged H-DNA sequences. The right terminal end of the C488 L-DNA is precisely defined by the beginning of an H-DNA repeat unit. Based on this definition, the unique L-DNA region had an average G+C content of 34.5% and a CpG dinucleotide frequency suppression rate of 0.32 (identical to A11) (Albrecht et al., 1992a). The 1458-bp repetitive terminal H-DNA had 69.9% G+C content (70.8% in A11).

Repetitive regions in the HVS A11 L-DNA occur in *orf48* and *orf73,* between *orf15* and *orf16,* and between *orf70* and *orf71* (Albrecht et al., 1992a). The location of all these repetitive sequences is conserved in HVS C488. ORF48 carries a large C-terminal domain consisting of repeated Gln and Asp residues which is clearly divergent between A11 and C488 (63.8% nucleotide conservation of ORF48; different arrays of Gln and Asp residues). In the genomes of Kaposi's sarcoma-associated herpesvirus (KSHV), Rhesus monkey rhadinovirus (RRV), alcelaphine herpesvirus type 1 (AHV-1), bovine herpesvirus type 4 (BHV-4), and murine gamma-herpesvirus 68 (MHV-68), a G+C-rich region is present between *orf70* and *orf71.* In BHV-4, this region is composed of a highly A+T-rich sequence of 750 bp, followed by two G+C-rich stretches of multiple direct repeats R2a and R2b; R2b is the major element of the replication origin identified within this region in BHV-4 (Zimmermann et al., 2001). However, in HVS and the closely related herpesvirus ateles (HVA) this region is devoid of these G+C-rich repetitive elements, but contains palindromic and A+T-rich sequences only (Albrecht, 2000; Lang and Fleckenstein, 1990). In HVS A11, the origin of lytic replication was mapped to this region (Schofield, 1994).

Coding capacity of the HVS C488 genome

Upon analysis of the C488 genome, ORFs were defined as previously done for other herpesviruses (Albrecht, 2000; Albrecht et al., 1992a; Ensser et al., 1997; Nicholas, 1996): (a) ORF size following an ATG codon larger than 60 aa; (b) presence of potential transcriptional start sites and polyadenylation sites; (c) high GENEMARK score (>0.5); (d) homology to previously described herpesviral or cellular genes. The longest of several overlapping ORFs was considered significant. By employing these criteria, we determined 77 potential ORFs as shown in Table 1 and Fig. 2A. Shorter ORFs, ORFs without ATG start codons, or ORFs derived from spliced messages would however be missed by this approach. The ORF nomenclature followed the rhadinoviral prototype sequence of the HVS strain A11 (Albrecht et al., 1992a).

Table 1
HVS ORFs and related ORFs in the rhadinoviruses HVA, KSHV, and RRV

ORF	Strand[a]	Start/exon1	Stop/exon2	*n* (aa)	kDa predicted	Signal peptide[b]	aa id (%)[c] HVS A11	aa id (%) HVA	aa id (%) RRV	aa id (%) KSHV	Description, homology with[d]
	c	1465	695	266	28.7		nh	32.8 to Tio	nh	nh	Tyrosine-kinase interacting protein, Tip
1	c	2091	1783	102	9.9		23.2 to StpA	40.0 to Tio	40.0 to ORF73 (GPP)	nh	Saimiri transformation-associated protein, StpC
2	c	5344	4703	213	24.6		80.5	nh	57.2	43.1	vDHFR
3	d	6602	10342	1246	138.5		93.6	70.1	nh; 25.4 to ORF75	nh; 26.9 to ORF75	vFGARAT
4a	d	10636	11718	360	40.6	20	61.4	60.3	34.2	34.1	Complement control protein homolog, membrane form
4b	d	10636/11497SD	SA11691/11740	303	33.8	20	57.3	57.6	ns	ns	Complement control protein homolog, soluble form
5	c	11952	11720	78	9.1		51.3	nh	nh	nh	
6	d	12310	15696	1128	127.6		97.5	86.5	54.3	55.0	Major single-stranded DNA binding protein, mDNA-BP
7	d	15703	17742	679	77.6		96.6	83.5	47.7	44.8	Processing and transport protein
8	d	17732	20158	808	91.8	19	96.5	86.0	55.4	56.0	Glycoprotein B
9	d	20232	23261	1010	114.2		97.8	87.7	62.6	62.3	DNA polymerase
10	d	23298	24521	407	45.2		98.8	82.6	25.6	25.4	Raji LF1 EBV
11	d	24524	25741	405	45.8		98.3	76.3	32.4	31.1	Raji LF2 EBV
12	c	26279	25770	169	19.4		90.5	nh	nh	26.6 to K3; 22.6 to K5	
13	c	27122	26667	151	17.2	22	98.0	nh	nh	nh	vIL-17
14	c	28140	27394	248	28.2	33	94.8	49.6	nh	nh	vSag
15	d	28946	29296	116	13.2	19	91.4	nh	nh	nh	vCD59
16	d	30036	30518	160	18.0		92.5	65.6	23.9	22.5	vBcl2
17	c	31972	30548	474	53.0		96.0	74.0	47.0	44.3	Protease/capsid protein, minor capsid scaffold protein
18	d	31965	32735	256	29.9		97.3	80.5	49.6	48.8	
19	c	34363	32732	543	61.3		97.8	79.6	47.9	45.4	Virion tegument protein
20	c	34904	33903	333	34.9		96.0	68.0	39.1	43.8	Fusion protein
21	d	34903	36486	527	60.0		96.0	75.2	34.8	31.7	Thymidine kinase
22	d	36483	38636	717	82.7	16	88.4	83.8	32.9	35.7	Glycoprotein H
23	c	39390	38629	253	28.9		99.2	82.0	31.7	36.9	
24	c	41589	39400	729	83.3		98.1	81.5	46.8	46.9	
25	d	41601	45716	1371	154.2		99.3	89.4	67.5	65.9	Major capsid protein
26	d	45732	46646	304	34.4		100.0	90.1	58.2	57.9	Capsid protein VP23
27	d	46653	47495	280	32.4		99.3	69.6	30.8	33.0	
28	d	47570	47785	71	7.6	35	82.4	73.2	22.4	32.4	
29	c	52932/52024SD	48940SA/47795	684	76.9		97.7	87.0	59.5	59.6	DNA packaging protein, terminase
30	d	48958	49185	75	8.3		94.7	82.7	33.3	33.3	
31	d	49152	49772	206	24.3		99.0	90.2	42.5	44.8	
32	d	49724	51049	441	50.9		97.1	71.7	34.5	33.2	
33	d	51042	52034	330	37.0		98.5	80.3	39.1	37.5	
34	d	52931	53881	316	36.2		98.1	87.0	43.3	43.5	
35	d	53868	54320	150	17.3		98.0	77.6	40.4	32.9	
36	d	54208	55503	431	48.8		98.8	81.6	29.4	32.2	Phosphotransferase, possible tyrosine kinase
37	d	55503	56954	483	55.6		99.0	87.8	53.0	50.5	Alkaline exonuclease
38	d	56909	57109	66	7.6		95.5	71.2	33.3	41.4	*N*-myristoylated in HSV
39	d	57134	58234	366	42.2	37	98.4	84.7	57.8	51.8	Glycoprotein M, integral membrane protein
40	d	58319	59671	450	51.9		95.6	69.2	30.5	28.1	Helicase–primase complex
41	d	59804	60289	161	18.7		96.3	75.0	33.1	31.1	Helicase–primase complex
40/41	d	58319/59638SD	SA59723/60289	628	72.6		95.9	70.2	—	—	Spliced in EBV (BBLF2+3)
42	c	61078	60281	265	30.0		98.5	80.8	41.2	41.2	
43	c	62735	61044	563	64.0		99.6	89.2	57.3	60.7	Minor capsid protein, virion protein
44	d	62710	65055	781	88.2		99.5	89.8	62.8	61.5	Helicase–primase complex, helicase
45	c	65869	65093	258	28.3		95.0	61.8	35.7	31.9	
46	c	66638	65880	252	28.9		95.2	85.3	58.7	59.4	Uracil DNA glycosylase
47	c	67032	66616	138	15.7	23	69.3	73.2	29.6	29.8	Glycoprotein L (CMV), *N*-myristoylation signals
48	c	69191	67038	717	83.9		63.8	51.9	31.9	28.8	Glu and Asp-rich, repetitive structure
49	c	70312	69401	303	35.8		94.7	73.7	25.6	22.3	
50a	d	69354/69374SD	SA70334/71875	520	58.3		70.6	ns	ns	ns	Rta homolog, exons 1+2
50b	d	70829	71875	348	38.5		70.4	71.2	28.9	27.0	Rta homolog, exon 2
51	d	72371	73264	297	33.2	17	39.0	38.2	ph	ph	Putative virus specific glycoprotein
52	c	73644	73297	115	13.1		62.3	66.0	33.9	32.2	
53	c	73958	73686	90	10.4	19	63.3	60.7	35.6	35.6	Putative glycoprotein N
54	d	74035	74898	287	32.3		94.1	74.2	39.4	33.9	Deoxyuridine triphosphatase, dUTPase

Table 1 (*continued*)

ORF	Strand[a]	Start/exon1	Stop/exon2	*n* (aa)	kDa predicted	Signal peptide[b]	aa id (%)[c] HVS A11	aa id (%) HVA	aa id (%) RRV	aa id (%) KSHV	Description, homology with[d]
55	c	75533	74931	200	22.3		99.5	84.0	48.7	47.5	
56	d	75512	78019	835	95.9		98.1	80.7	43.9	45.4	Helicase–primase complex, DNA-replication, primase
57	d	78086/78134SD	SA78224/79461	427	48.0		98.6	ns	ns	26.3	IE52, spliced form
57b	d	78301	79458	385	43.4		98.7	75.1	34.7	29.2	Exon2 of IE52
58	c	80879	79806	357	40.6		98.3	83.0	30.6	28.5	
59	c	81991	80876	371	40.7		95.1	74.9	37.7	36.1	Processivity factor, subunit of DNA-polymerase
60	c	83019	82102	305	35.1		98.7	91.8	62.5	64.9	Ribonucleotide reductase, small subunit
61	c	85328	83025	767	87.3		97.4	89.8	54.0	53.4	Ribonucleotide reductase, large subunit
62	c	86320	85328	330	37.4		99.1	85.2	42.8	39.8	Probable capsid assembly and DNA maturation protein
63	d	86327	89023	898	103.3		97.8	75.9	35.4	32.0	Legument protein
64	d	89026	96459	2477	281.2		94.4	72.0	32.3	31.7	Large tegument protein
65	c	96879	96463	138	15.2		70.5	61.9	39.9	35.5	Capsid protein
66	c	98179	96872	435	50.3		97.9	80.8	35.4	37.4	
67	c	98799	98092	235	26.9		97.4	84.4	51.8	53.8	Tegument protein
67.5	c	99041	98796	81	9.2		98.8	85.0	50.0	41.6	After frame shift correction in strain A11 (G inserted after pos. 99117)
68	d	99046	100356	436	49.1		97.7	78.0	49.3	45.6	Probable major envelope glycoprotein
69	d	100359	101144	261	29.8		99.2	88.8	51.0	54.2	
70	c	102267	101383	294	33.5		98.0	85.5	65.6	66.0	Thymidylate synthase
71	c	104988	104485	167	19.2		94.0	44.8	14.5	20.0	vFLIP, FLICE interacting protein, inhibitor of apoptosis
72	c	105753	104989	254	28.6		98.4	74.0	31.3	34.8	vCyclin
73	c	107314	105809	501	55.6		76.1	44.2	21.3	30.7	LANA homolog, repetitive structure
74	d	107813	108877	354	37.1		96.0	70.1	33.6	35.9	vGPCR, IL-8 receptor
75	c	112761	108862	1299	143.3		95.7	73.9	35.5	36.9	Virion protein, vFGARAT

[a] d, ORF encoded on direct strand; c, complementary strand

[b] Signal peptide length predicted by SignalP

[c] Amino acid identity calculated with the GCG program GAP (standard parameters, gap creation penalty 8, gap extension penalty 2).

[d] For comparison see Albrecht (2000); Albrecht et al. (1992a); Alexander et al. (2000); Russo et al. (1996); Searles et al. (1999). ns, no splicing data available; nh, no homolog; ph, positional homolog in the respective virus.

Within the L-DNA, HVS C488 encodes at least 77 ORFs and 5 URNA genes. The conserved herpesviral ORFs are arranged in four gene blocks that separate the five regions containing genes which are conserved among the rhadinoviruses and not found in other herpesviruses. At least 10 viral genes share homology with cellular genes, and most of these C488 genes were found to be highly conserved in A11. The only gene absent in the prototype strain A11 was the transformation-associated *tip* gene, next to the positionally conserved transforming *stp*. Thus, the major difference between HVS C488 and A11 are the two genes *stpC* and *tip* instead of *stpA* and the fact that HVS C488 carries only five (HSUR 1, 2, 5, 4, and 7) instead of seven URNA transcription units of strain A11 (Albrecht et al., 1992a; Biesinger et al., 1990).

In contrast to *stpC* and *tip*, which are essential for transformation and pathogenicity (Duboise et al., 1998b; Knappe et al., 1997), the C488 URNAs were dispensable for the in vitro transformation of simian and human T cells (Ensser et al., 1999). The EBERs of EBV are also dispensable for B cell transformation (Swaminathan et al., 1991), although recent work suggested that they may promote tumorgenicity of EBV-negative Burkitt lymphoma cells (Komano et al., 1999; Ruf et al., 2000). In this genomic region, the divergence between different HVS strains appears to be in the same order of magnitude as between HVS and HVA. Instead of StpC and Tip, HVA encodes the protein Tio (two-in-one) expressed from a spliced transcript. Tio combines N-terminal motifs reminiscent of the StpC collagen repeats with the SH3B and CSKH regions of Tip. Similarly to the HVS oncoproteins StpA, StpB, and Tip, Tio binds to and is phosphorylated by Src-family tyrosine kinases Lck, Src, and Fyn. HVA carries only two URNA genes which seem to be homologs to HSUR1 and HSUR2, while a dehydrofolate reductase (DHFR) gene is missing (Albrecht, 2000; Albrecht et al., 1999).

Besides the transformation-associated region, most other ORFs were highly conserved between C488 and A11 with amino acid identities exceeding 90%. However, the predicted protein sequences of ORF4 (complement control protein homolog, CCPH; 61.4% aa identity), ORF5 (51.3%), ORF22 (gH; 88.4%), ORF28 (82.4%), ORF47 (gL; 69.3%), ORF48 (repetitive sequence; 63.8%), ORF50 (R-transactivator, Rta; 70.6%), ORF51 (glycoprotein; 39.0%), ORF52 (62.3%), ORF53 (putative gN; 63.3%), ORF65 (capsid protein; 70.5%), and ORF73 (homolog to

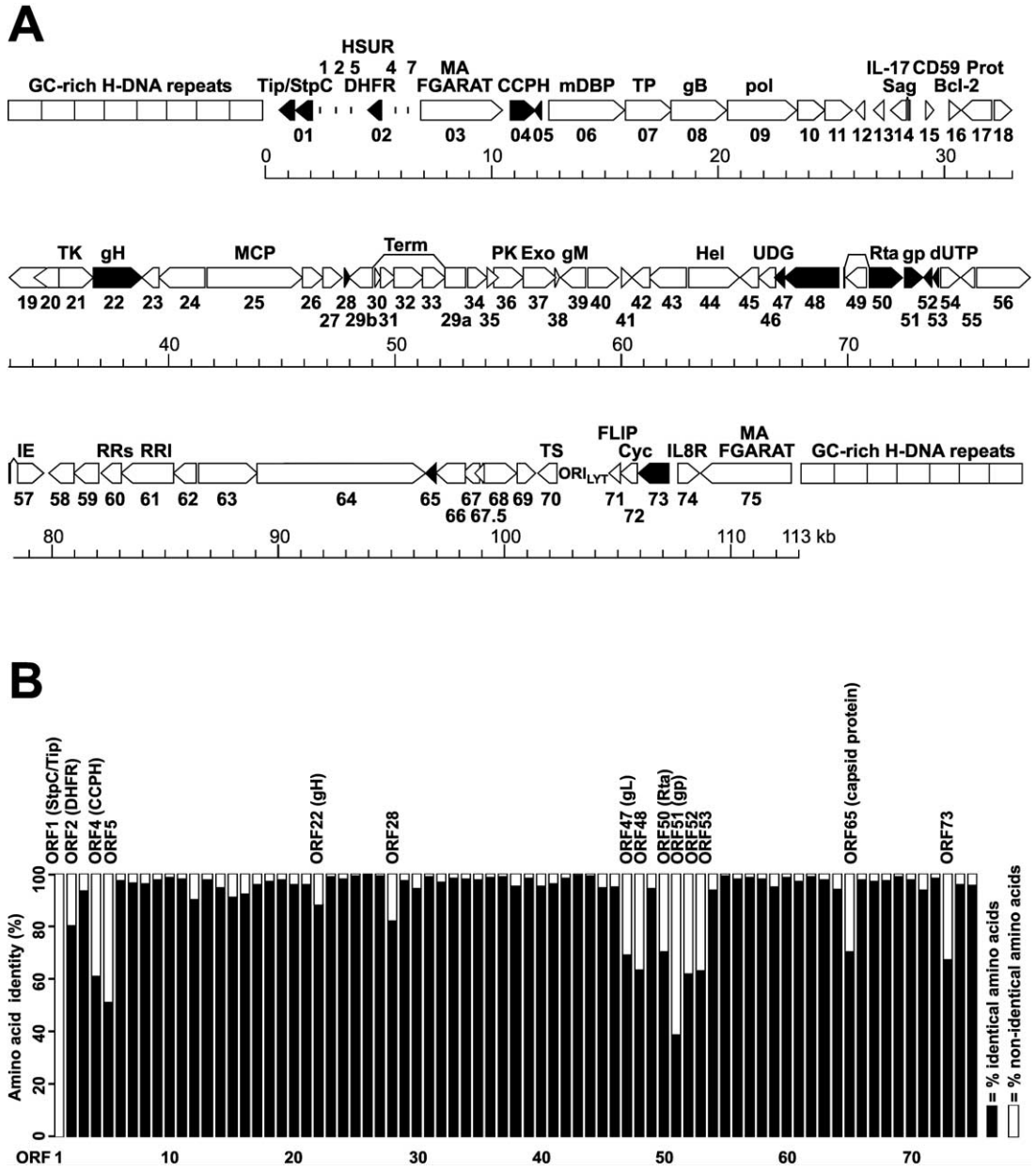

Fig. 2. (A) HVS genome organization. Genes with more than 10% aa divergence between the HVS strains A11 and C488 are marked in black. (B) Amino acid conservation of the individual HVS reading frames between the strains A11 and C488. Each bar represents one viral open reading frame. Proteins with significant divergence are indicated by name.

the latent nuclear antigen of KSHV, LANA, repetitive sequence; 76.1%) are more variable.

Viral glycoproteins

The analysis of the HVS C488 ORFs with the programs PSORT II and SignalP (Nakai and Horton, 1999; Nielsen et al., 1997) revealed that 11 predicted proteins contain putative eukaryotic signal sequences at their N-terminus (Table 1). Further comparison with conserved amino acid motifs from the PROSITE database (Hofmann et al., 1999) showed that most of these ORFs also contained N-glycosylation sites (N-X-T/S). ORF8, ORF22, ORF39, and ORF47 are the HVS sequence homologs to the glycoproteins gB, gH, gM, and gL of herpes simplex virus (HSV) and cytomegalovirus (CMV), respectively. A small glycoprotein encoded by ORF53 (90 aa) has homologs in HVS A11, KSHV, RRV, MHV-68, AHV-1, and EBV and may correspond to gN of α- and β-herpesviruses. The glycoproteins ORF4/CCPH and ORF15/vCD59 are functional viral homologs to complement regulatory proteins (Albrecht and Fleckenstein, 1992; Albrecht et al., 1992b; Fodor et al., 1995; Rother et al., 1994), and ORF13 and ORF14 are homologs to the glycoprotein IL-17 and to murine superantigens (Sag)

Table 2
In vitro transformation of T cells from human and *Saguinus oedipus* with new HVS isolates.

Saimiri sciureus no.	From colony no.	HVS subgroup	T cell transformation			
			Human		*Saguinus oedipus*	
			CB-84	CB-94	B240	R178
6045	1	B	−[c]	−	Delayed	Delayed
6051	1	A + (C)[b]	−	−	+	+
8907	1	C	Delayed	Delayed	+	+
5746	2	C	+	Delayed	+	+
5747	2	A + (C)	−	−	+	+
5753	2	C	+	+	+	+
5947	3	C	Delayed	+	+	+
5945	3	C	+	+	+	+
5952	3	C	Delayed	+	+	+
6661	1 × 2[a]	(A) + C	+	+	+	+
6355	2 × 3	A	−	−	+	+
C488	—	C	+	+	+	+

[a] Parents were from different colonies.
[b] In case of double infections, the less dominant subgroup is given in parentheses.
[c] −, transformation was not achieved; +, successful transformation experiment.

(Duboise et al., 1998a; Knappe et al., 1997, 1998a,b; Nicholas et al., 1990; Yao et al., 1995).

The putative type I transmembrane glycoprotein ORF51 has weakly conserved, but positionally equivalent, Ser- and Thr-rich homologs in many γ-herpesviruses. The EBV homolog gp350/220 mediates attachment to the EBV receptor on B cells (CD21) (Fingeroth et al., 1984). The MHV-68 homolog M7 encodes gp150 that is expressed in infected cells and present in virions. Antisera to gp150 neutralize virion-associated infectivity, and the mononucleosis-like syndrome is prevented by immunization with recombinant gp150 vaccinia vectors (Stewart et al., 1996, 1999). AHV-1 A8 and BHV-4 BORF D1 encode similar glycoproteins (Ensser et al., 1997; Lomonte et al., 1995; Zimmermann et al., 2001). The major immunogenic glycoprotein of KSHV is encoded by K8.1 from a spliced transcript and is included in the viral envelope (Li et al., 1999; Raab et al., 1998). The RRVs also contain the equivalent R8.1 (Alexander et al., 2000; Searles et al., 1999). Interestingly, the glycoprotein R8.1 was highly conserved between the RRVs, as was K8.1 between different KSHV isolates, whereas HVS isolates of different subgroups show the most pronounced glycoprotein sequence divergence in ORF51 (61%). The putative membrane glycoprotein encoded by HVS ORF51 is a candidate for the interaction with a specific host cell receptor. The positional homologs in the rhadinoviruses BHV-4, RRV, and KSHV are encoded by spliced transcripts; RT-PCR experiments indicated that, similarly to the situation in MHV-68, the HVS ORF51 is derived from an unspliced message (data not shown).

Among the HVS genes with pronounced interstrain variation were several glycoproteins, namely ORF4/CCPH, ORF22/gH, ORF28, ORF47/gL, ORF51, and ORF53/gN. A similar situation was observed for the genomes of the two sequenced rhesus rhadinovirus isolates where ORF4/CCPH, ORF22/gH, and ORF47/gL also showed a high degree of sequence variation (Alexander et al., 2000; Auerbach et al., 2000; Searles et al., 1999). The selective pressure exerted by the host immune system induces diversity of the immunogenic glycoproteins. The significant sequence variability of these immunogenic proteins may relate to the fact that squirrel monkeys can be infected with different HVS isolates at the same time (Desrosiers and Falk, 1982; Fickenscher et al., 1997; Medveczky et al., 1984, 1989), which was also observed in this study (Table 2, Fig. 5B).

Conservation of cellular homologs

A series of HVS genes was previously recognized as pirated homologs of cellular genes. These cell-homologous genes are localized to the left and right ends of the L-DNA genome. At the left terminus, such genes flank a conserved block of typical herpesvirus genes (ORF6 to ORF11), ending with ORF16/vBcl-2. The respective region at the right L-DNA end starts with ORF70/thymidylate synthase. Thus, the terminal regions of the γ-herpesvirus genomes are integration sites for cellular genes, probably facilitated by the viral replication that was described to originate in these genome regions (Kung and Medveczky, 1996; Schofield, 1994). Most of the cell-homologous proteins are highly conserved between the HVS strains A11 and C488, among them ORF13/vIL-17, ORF14/vSag, ORF16/vBc12, ORF71/vFLIP, ORF72/vCyclin, ORF74/vIL-8 receptor, and enzymes of nucleotide metabolism, namely ORF21/thymidine kinase, ORF54/dUTPase, ORF70/thymidylate synthase, and ORF3 and ORF75, the two homologs to *N*-formylglycineamide ribotide amidotransferase (vFGARAT). ORF2 (DHFR) is localized in the highly divergent transformation-associated genomic L-DNA region and is only 80.5% conserved. Recently, ORF12 was described to show a limited

similarity to the K3 and K5 proteins of KSHV which down-regulate MHC class I surface expression and block the susceptibility to natural killer cells (Coscoy and Ganem, 2000; Ishido et al., 2000a,b). In addition, homologies to HVS ORF12 are found in BHV-4, in poxviruses (r153R of rabbit fibroma virus and VC07 of swine poxvirus), and in the human genome (NP_057580, NP_060393). ORF12 of HVS C488 was dispensable for replication and T cell transformation (Knappe et al., 1998a). Whereas the complement regulator ORF15/vCD59 is almost identical, the secreted or membrane-bound ORF4/CCPH shows the highest variability in the group of cell-homologous genes between the strains A11 and C488 (38.6 or 42.7%, respectively). Previously, we showed that the genes encoding vIL-17 and vSag, as well as the vFLIP and the vCyclin genes are dispensable for in vitro transformation and pathogenicity in cottontop tamarins (Ensser et al., 2001; Glykofrydes et al., 2000; Knappe et al., 1997, 1998a,b). Altogether, the high degree of conservation of the cell-homologous viral proteins and the results with the respective deletion viruses indicate that they are not involved in the T cell transformation process or at least cannot explain a differential transforming phenotype of HVS strains. However, the high conservation level suggests an important role in the viral replication and persistence in the natural host species where HVS is an apathogenic passenger circumventing the squirrel monkey's immune defense.

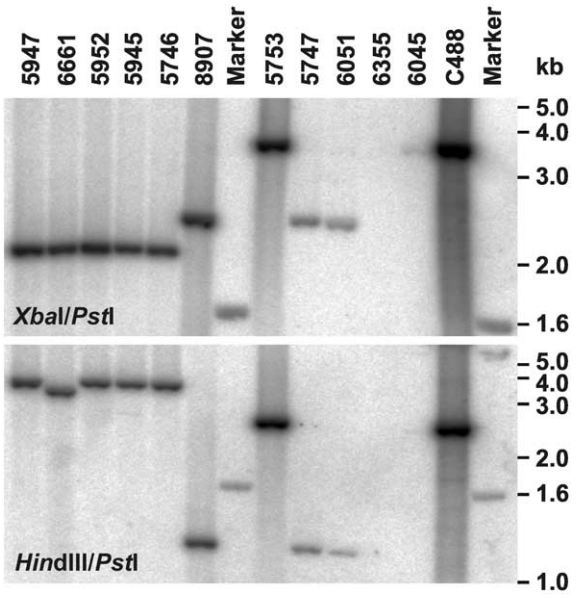

Fig. 3. Restriction fragment length polymorphism of HVS subgroup C isolates. Virion DNA of new virus isolates (Table 2) and strain C488 was cleaved with either *Xba*I/*Pst*I or *Hin*dIII/*Pst*I and subsequently analyzed by Southern blot hybridization with an *stpC*-specific radioactive probe. In accordance to the PCR-based subgroup assignment, weak signals were also observed with the isolates 5747 and 6051, which simultaneously contained a dominant subgroup A strain.

The positional homolog to the latent nuclear antigen of KSHV

In KSHV, ORF73 is the major latent nuclear antigen which was suggested to be involved in the plasmid replication of persisting virus genomes (Ballestas et al., 1999). However, *orf73* expression in HVS-transformed human T cells is not detectable by Northern blot analysis (Fickenscher et al., 1996; Knappe et al., 1997), although *orf71* and *orf72*, which are located on the same transcript, are detectable by more sensitive RT-PCR (Glykofrydes et al., 2000). The HVS C488 ORF73 protein (501 aa) is smaller than KSHV ORF73/LANA (1089 aa), but considerably larger than the HVS A11 counterpart (407 aa). HVS C488 ORF73 is able to maintain the latent state of HVS in permissive cells by suppressing ORF50 R-transactivator expression (Schäfer et al., 2003). ORF73 is characterized by a highly repetitive and highly charged central region containing Glu-Arg, Glu-Gly, and Glu-Ala motifs. The N-terminus contains nuclear localization signals similar to A11 ORF73 (Hall et al., 2000), and the C-terminal region resembles the DNA-binding domain of EBNA-1 of EBV (Bochkarev et al., 1995). Whereas the ORF73/LANA proteins of two RRV isolates are highly conserved, the ORF73 sequences of the HVS A and C strains diverge considerably (23.9%; Table 1). The variability of ORF73 between different rhadinovirus species is high; however, the N- and/or *C*-terminal sequences are better conserved. The Pro-rich sequences in the N-terminal ORF73 regions of equine herpesvirus 2 (EHV-2), MHV-68, RRV, and KSHV are missing in HVS and HVA. In contrast, the extensive Gly-Glu/Asp and Gly-Arg domains of KSHV, HVS, HVA, or EHV-2 are lacking in RRV and MHV-68.

Viral regulatory genes

The HVS genome contains only a few genes which may have regulatory functions for virus replication. One immediate-early gene, *orf14*, is a superantigen homolog, but was ruled out to be involved in replication (Duboise et al., 1998a; Knappe et al., 1997, 1998b). The immediate-early regulatory protein ORF57 of C488 is highly conserved, and ORF57 of strain A11 was shown to repress the expression of spliced viral transcripts at a posttranscriptional level. ORF57 is a nucleocytoplasmatic shuttle protein which co-localizes with the splicing factor SC-35, binds to importins, and regulates both *orf50a* and its own transcription at a posttranscriptional level (Cooper et al., 1999; Goodwin and Whitehouse, 2001; Goodwin et al., 1999, 2000; Whitehouse et al., 1998b). An observed N-terminal sequences divergence of the HVS ORF57 proteins was resolved by removing an erroneous nucleotide (position 78,288) in the A11 genome (Albrecht et al., 1992a). Although there is considerable sequence variability between *orf57* of HVS and KSHV, the splicing pattern is conserved (Bello et al., 1999; Kirshner et al., 2000).

orf50 encodes a regulatory protein homologous to

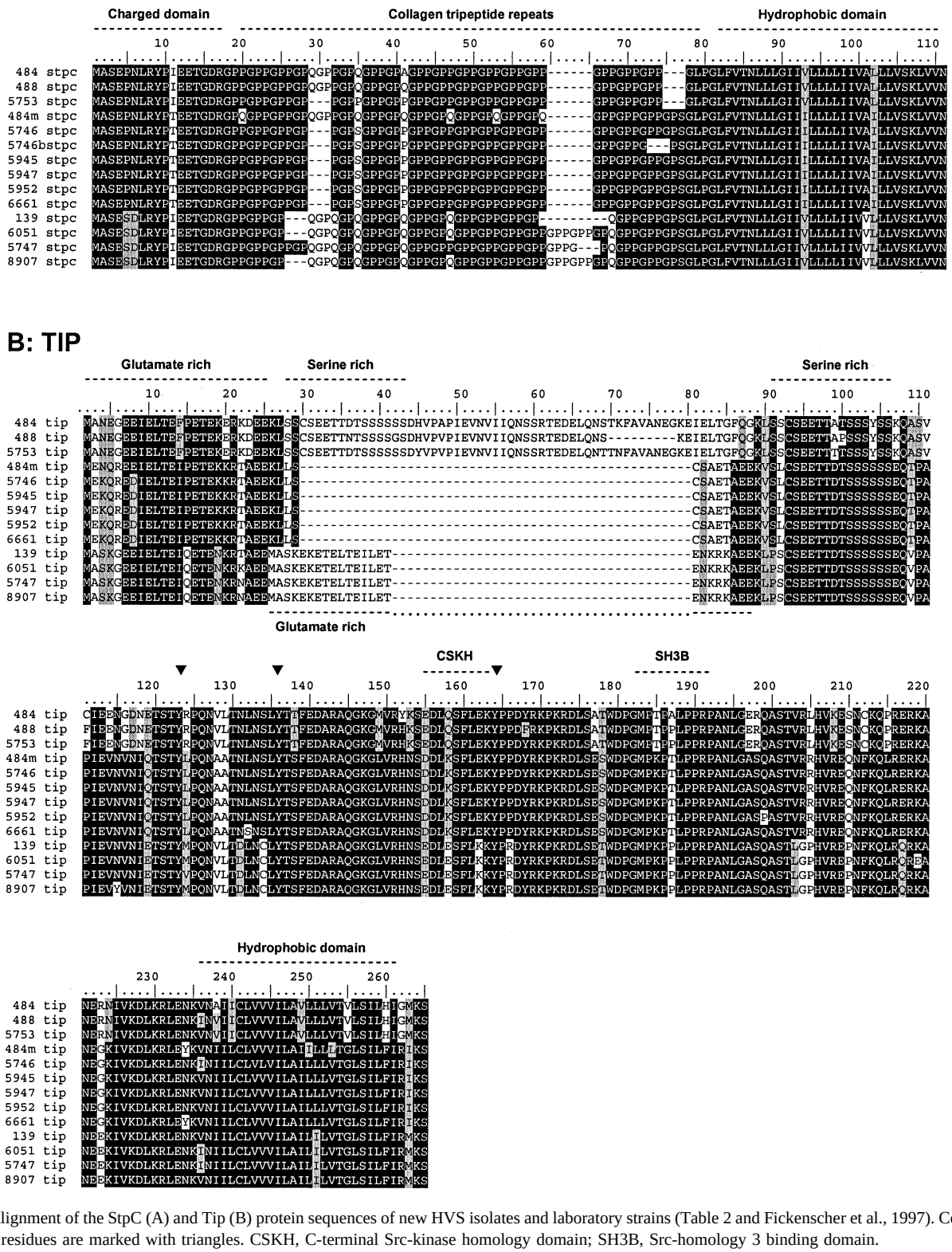

Fig. 4. Alignment of the StpC (A) and Tip (B) protein sequences of new HVS isolates and laboratory strains (Table 2 and Fickenscher et al., 1997). Conserved tyrosine residues are marked with triangles. CSKH, C-terminal Src-kinase homology domain; SH3B, Src-homology 3 binding domain.

BRLF-1 or the R transactivator (Rta) of EBV (Chang et al., 1998; Liu et al., 1996). The HVS *orf50/Rta* gene is expressed in two forms. The larger ORF50A protein is translated from a spliced transcript, and the smaller C-terminal ORF50B is derived from shorter transcripts initiating downstream of the *orf50a* splice acceptor site. A11 ORF50A transactivated transcription from early virus gene promoters such as the major DNA binding protein (*orf6*) promoter,

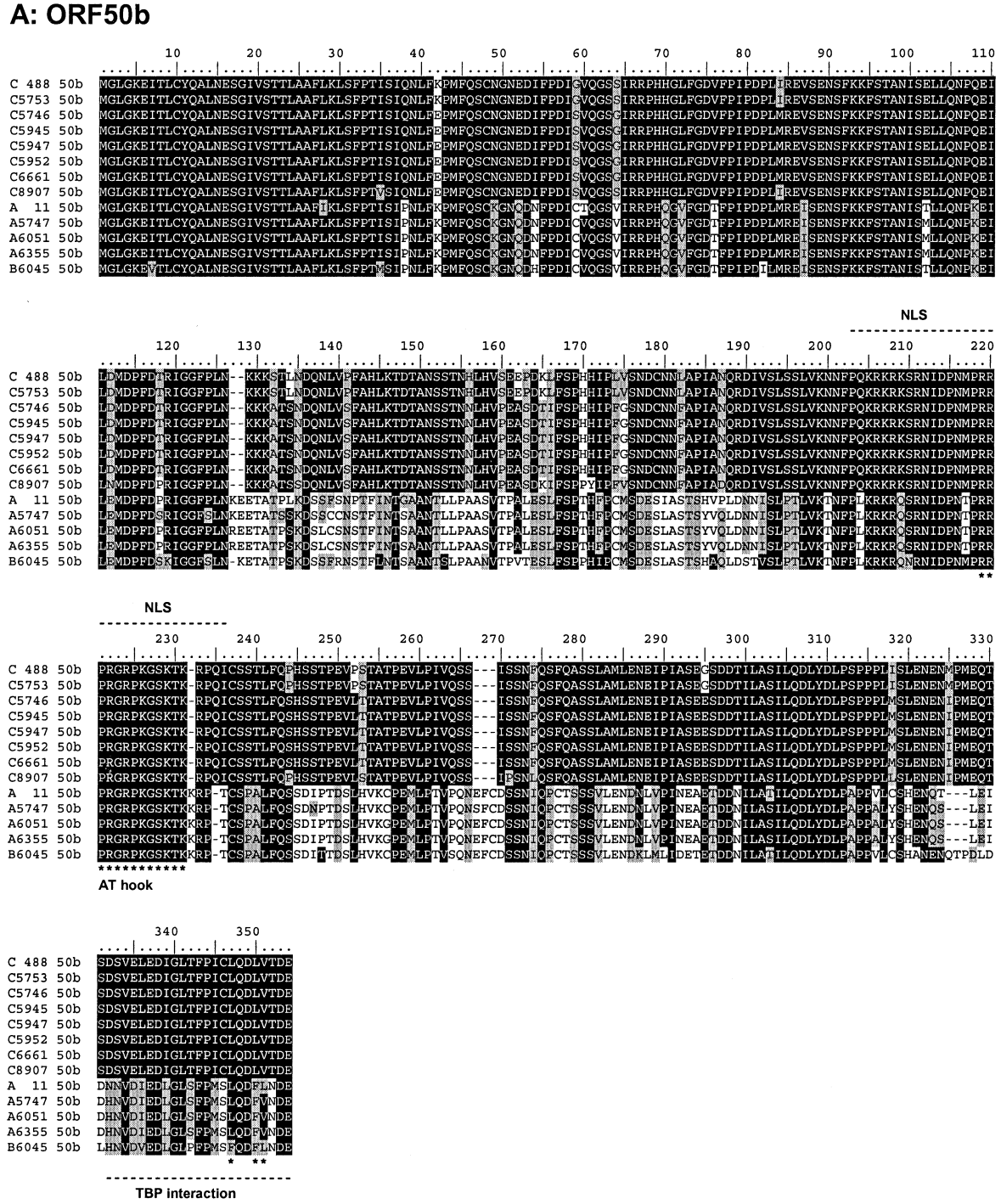

Fig. 5. Alignment of variable protein sequences of new HVS isolates and laboratory strains (Table 2). (A) ORF50b alignment. NLS, bipartite nuclear localization signal, predicted with PSORT II (Nakai and Horton, 1999); AT-hook, putative high-affinity DNA-binding AT hook type I motif of minor groove DNA binding proteins such as high mobility group I-C protein (Aravind and Landsman, 1998). TBP, the interaction site with TATA binding protein (TBP) contains three essential amino acids (*) which are conservatively exchanged (Hall et al., 1999). (B) ORF51 alignment. Signal peptide and transmembrane domains, predicted with SignalP and TMHMM (Nielsen et al., 1997) *, *N*-glycosylation sites (top for C488, bottom for A11).

whereas A11 ORF50B had a strongly reduced transactivation function (Whitehouse et al., 1997a, 1997b; 1998a). However, in contrast to strain A11, C488 ORF50B showed full transactivation capability (Thurau et al., 2000). Only ORF50A, and not ORF50B, of both HVS strains A11 and C488 reactivated lytic virus replication in persistently infected A549 human lung carcinoma cells (Goodwin et al., 2001). In the C-terminal portion, all ORF50 forms carry a bipartite nuclear localization signal which includes a typical high-affinity DNA-binding AT hook type I motif as con-

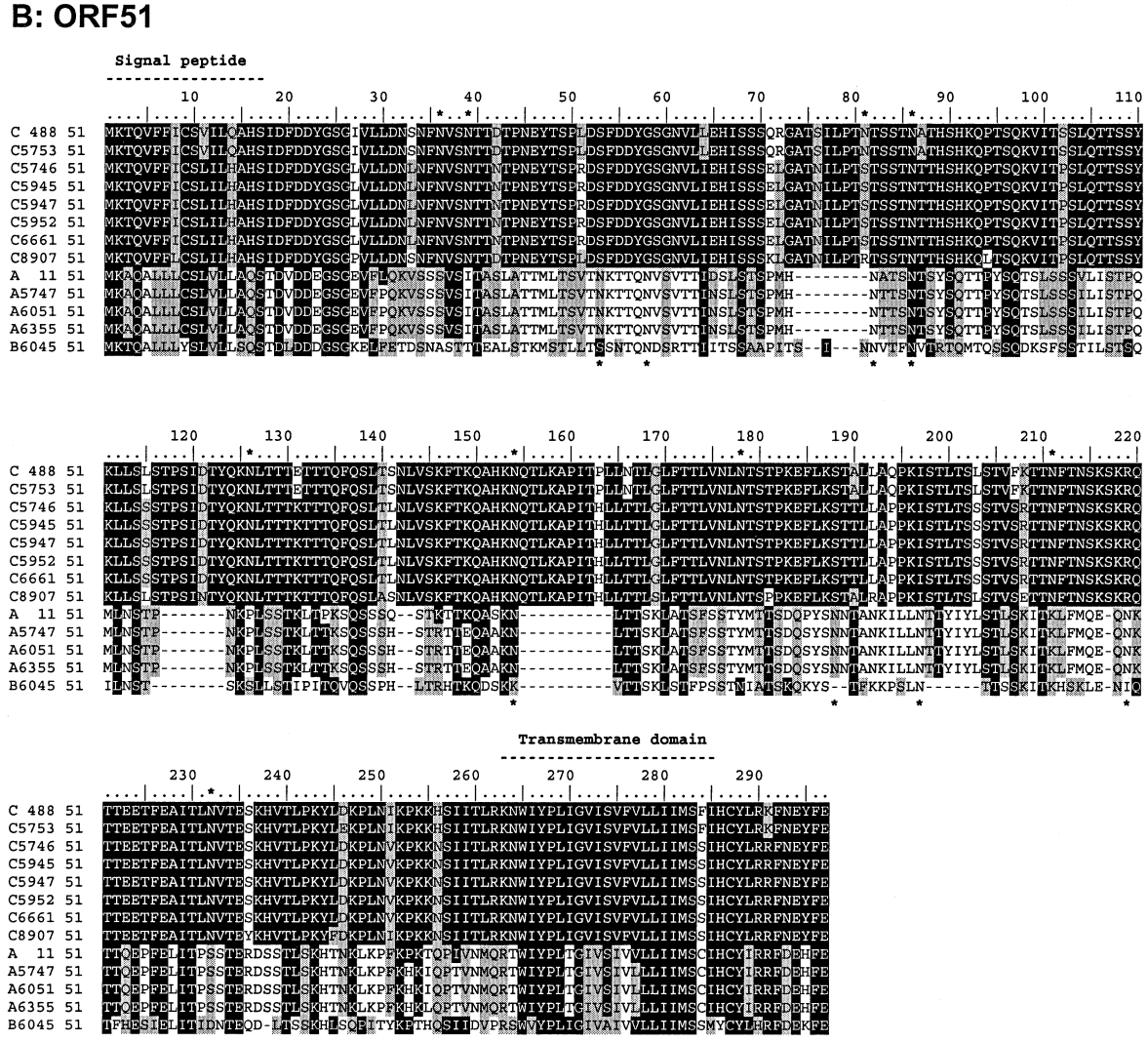

Fig. 5 (*continued*)

tained in minor groove DNA binding proteins such as the high-mobility group I-C protein (Aravind and Landsman, 1998). The C-terminus of the ORF50 proteins shows conservative exchanges within the activation domain which interacts with TATA binding protein (Fig. 5A) (Hall et al., 1999). In contrast to the highly conserved ORF57, HVS C488 ORF50 is one of the most variable proteins. KSHV ORF50 selectively upregulates delayed-early viral genes, disrupts latency, and induces the lytic gene expression cascade in latently infected B cells (Lukac et al., 1998, 1999). In cells dually infected with EBV and KSHV, each Rta activated only the autologous lytic cycle genes (Sun et al., 1998). KSHV ORF50 binds to the cellular CREB protein and histone deacetylase, which modulates ORF50-activated viral transcription (Gwack et al., 2001). Similarly, MHV-68 ORF50 transactivates the *orf57* promoter, induces the expression of early and late genes, the lytic replication of viral DNA, and the production of infectious viral particles (Liu et al., 2000; Wu et al., 2000).

Sequence divergence of 11 new HVS isolates

The results from the genome comparison of two virus strains of the same species can be strongly biased if the viruses accumulate mutations during long-term passage in cell culture. This problem is largely excluded for the strain C488 which was passaged only for a few times from a sample frozen early after isolation (Fickenscher et al., 1997). However, for strain A11 the passage history is unclear. To exclude artifacts and to obtain more knowledge on the variability of HVS strains, we isolated and analyzed 11 new HVS strains from three different squirrel monkey (*S. sciureus*) colonies of the German Primate Center in Göttingen (Greve et al., 2001). Using *stp* allele-specific DNA PCR (Hör et al., 2001), these isolates were assigned to the three HVS subgroups (Table 2) (Medveczky et al., 1984, 1989). In three cases, a coinfection of the same animal with viruses from different subgroups was detectable, similar to other cases in the literature (Desrosiers and Falk, 1982; Ficken-

scher et al., 1997; Medveczky et al., 1984, 1989). The nine subgroup C isolates showed a restriction length polymorphism in the left-terminal L-DNA region coding for the StpC and Tip oncoproteins (Fig. 3). The transformation-associated regions of the new subgroup C viruses were sequenced and compared with the previously known alleles (Biesinger et al., 1990; Fickenscher et al., 1997; Geck et al., 1990). Whereas only minor variations were observed in the StpC proteins (Fig. 4A), the 13 available Tip protein sequences could be assigned to three families (Fig. 4B) with the prototype viruses C488, C484(M), and C139, as suggested in a previous study (Fickenscher et al., 1997).

Since we had already observed a pronounced structural and functional variation of the *orf50* genes between strains A11 and C488 (Thurau et al., 2000), the entire *orf50* regions were amplified and the sequences from the 11 new isolates (three subgroup A, one subgroup B, and seven subgroup C sequences) were compared with the A11 and C488 alleles. The most pronounced diversity was observed for *orf50b* and *orf51* (Fig. 5). The whole region occurs in two alleles: one allele is specific for subgroup A and B; the other allele is specific for subgroup C strains. Laboratory strain-specific artifacts can be excluded, as the whole region occurs in two alleles within the prototype A11, C488, and the new isolates. The capability of transforming human T cells in culture strictly correlated with the assignment to subgroup C (Table 2). Thus, different alleles of *orf50* and *orf51* seem to strictly segregate with the transforming genes of the respective subgroup. A contribution of this segregation of *orf50* and *orf51* to the phenotypic differences between the subgroups remains to be investigated. Subgroup C viruses might have a stronger affinity to their receptor on human T cells due to their specific ORF51 protein. Thus, besides the transformation-associated region, the variability of HVS strains localizes mainly to proteins with repetitive motifs (ORF48, ORF73), to the capsid protein ORF65, to several glycoproteins (ORF4, ORF22, ORF28, ORF47, ORF51, ORF53), and to the transactivator ORF50/Rta. This variability evolved in the natural host, the squirrel monkey, and might afflict primary and persistent infection. However, since subgroup C strains of HVS have the unique capability of transforming human T cells in culture, the functional contribution of these additional genes to the transforming phenotype remains a relevant question.

Materials and methods

Cell lines and virus culture

Owl monkey kidney (OMK) cells (ATCC CRL1556) (Daniel et al., 1976) were propagated in Dulbecco's minimal essential medium with 10% fetal calf serum, 100 μg/ml gentamycin, and 350 μg/ml L-glutamine. HVS strain C488 (Biesinger et al., 1990; Desrosiers and Falk, 1982) was propagated for a few passages by infection of fresh OMK cells with supernatant from infected cells showing severe cytopathic changes. For virion preparation, the supernatant was precleared from cellular debris by centrifugation at 2000 *g*. Virions were then sedimented by ultracentrifugation and purified on sucrose density gradients. After lysis of the virions, the virus DNA was prepared by equilibrium CsCl density gradient ultracentrifugation (Fickenscher and Fleckenstein, 2002).

Pulse-field agarose gel electrophoresis

Clamped homogenous electric field electrophoresis was performed with 1% fast-lane agarose gels (FMC, Biozym, Hessisch-Oldendorf, Germany) in 0.5× TBE buffer with a CHEF DR-III System (Bio-Rad, Munich, Germany) set to 6 V/cm at an angle of 120° for 14 h, with switch time incrementing linearly from 2 to 20 s. Approximately 0.5 μg of gradient purified virion DNA was loaded per lane along with the midrange marker I (New England Biolabs, Frankfurt, Germany).

Isolation of HVS from squirrel monkeys

Blood samples (1–2 ml) were obtained from 11 squirrel monkeys of three different colonies at the German Primate Center, Göttingen. The peripheral blood mononuclear cells (PBMC) were prepared by direct centrifugation of EDTA blood. The erythrocytes were lysed by treatment with ACK buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.3) for 5 min followed by extensive washing in phosphate-buffered saline. Freshly isolated PBMC (approximately 10^6 cells) from each animal were cocultivated with permissive OMK cells to isolate the virus. After a typical cytopathic effect had led to cell lysis, the virus isolates were assigned to the subgroups by DNA PCR (Hör et al., 2001) for the respective transformation-associated genes *stpA*, *stpB*, and *stpC*. The 11 virus isolates were tested for their capability of transforming human (two cord blood donors) and cottontop tamarin T cells (donors B240 and R178) (Greve et al., 2001; Knappe et al., 1998a, 1998b).

Virions were concentrated by centrifugation from 1 ml of virus supernatant. The virion DNA was released in sodium dodecyl sulfate buffer in the presence of proteinase K followed by phenol/chloroform extraction and ethanol precipitation. Virion DNA was cut with either *Xba*I/*Pst*I or *Hind*III/*Pst*I and analyzed by Southern blot hybridization with an *stpC*-specific DNA probe. The terminal transformation-associated and the variable *orf50* regions of the new isolates were amplified by PCR with Taq polymerase from virion DNA and sequenced from at least two independent clones. In the rare case of divergence between these two sequenced clones, additional independent clones and PCR products were sequenced. The sequence readings were electronically processed as described below. The *stpC/tip* region sequence contigs comprised 1540 nt in average with a

redundance of 7.06 and the *orf50* region contigs comprised 3866 nt in average with a redundance of 8.38.

Cloning of the C488 genome

Purified genomic virion DNA was digested with the restriction enzymes *Hind*III or *Xba*I and cloned as small fragments into the plasmid vector pBluescript KS+ (Knappe et al., 1997). For subcloning of larger fragments into cosmid vectors, 2 μg of viral DNA was partially digested with the restriction enzyme *Sau*3AI, resulting in an average fragment size of 30 to 60 kb as judged by analytical pulse-field gel electrophoresis (Ensser et al., 1999). These fragments were ligated to 1 μg of dephosphorylated *Bam*HI-cut cosmid vector pWE15 (Stratagene, Amsterdam, The Netherlands) and packaged into lambda phage particles (Gigapack II Gold, Stratagene). The packaged cosmids were transduced into *Escherichia coli* JM109 cells grown in LB medium with 0.2% maltose and 10 mM $MgSO_4$. Colonies from LB agar plates with 50 μg/ml ampicillin were subsequently screened by small-scale alkaline lysis, restriction enzyme mapping, pulse-field gel electrophoresis, and sequencing of the cosmid insert ends. Large-scale preparation of cosmid DNA was done by alkaline lysis and anion exchange chromatography or CsCl-equilibrium gradient ultracentrifugation. Insert DNA from right-terminal L-DNA region (cosmids Dc5 and cos40) was sheared by sonification to fragments of 1–3 kb; the DNA ends were blunted with Klenow and T4-DNA polymerase and cloned into the vector pCRScript (Stratagene). Plasmid and cosmid DNA from *E. coli* DH5α or DH10B was sequenced using either ABI 373A or 377 PRISM analyzers and the Taq dye deoxyterminator chemistry (Applied Biosystems, Weiterstadt, Germany). Oligonucleotides were synthesized by Eurogentec (Seraing, Belgium) or Sigma-ARK (Darmstadt, Germany). The sequence readings were assembled with the program XBAP (Dear and Staden, 1991) on a Sparcstation10 (SUN Microsystems, Mountain View, CA). Oligonucleotides were selected using the program OSP (Hillier and Green, 1991) implemented in XBAP.

Nucleotide and protein sequence analysis

The GCG package (version 7, Devereux et al., 1984) with the programs FASTA (Pearson and Lipman, 1988) and BLAST (Altschul et al., 1990; Gish and States, 1993) were used for analysis of nucleotide and amino acid sequences and for comparison with the GenBank and Swissprot databases (versions 113.0 and 37.0, respectively). Potential coding regions were identified with the program GENEMARK (Borodovsky et al., 1994) using matrices for eukaryotic (human) and γ-herpesviral DNA (kindly compiled by William Hayes, Georgia Institute of Technology, Atlanta, GA). Putative signal sequences and protein structure were analyzed by the neuronal network-based SignalP trained on eukaryotic sequences (Nielsen et al., 1997), by PSORT II (Nakai and Horton, 1999), and by comparison to the PROSITE database (Hofmann et al., 1999). Protein and nucleotide sequences were aligned with the programs ClustalW and BioEdit (T. Hall, North Carolina State University, Raleigh, NC). The complete L-DNA sequence of HVS C488 is deposited under the EMBL Accession No. AJ410493; the terminal H-DNA repeat sequence is deposited under AJ410494. The individual sequences of the *stpC/tip* regions and *orf50* regions of the new HVS isolates were assigned to the EMBL Accession No. AJ410475 to AJ410481 and AJ410482 to AJ410492, respectively.

Acknowledgments

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