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The Superantigen-Homologous Viral Immediate-Early Gene *ie14/vsag* in Herpesvirus Saimiri-Transformed Human T Cells

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Herpesvirus saimiri C488 transforms human T lymphocytes to stable growth in culture. The growth-transformed human T cells harbor the viral genome in a nonintegrated episomal form without production of virus particles. In these cells, virus gene expression was previously found to be confined to the transforming genes *stpC* and *tip*. In order to analyze virus gene expression in more detail, we applied a subtractive hybridization technique and compared stimulated virus-transformed cells with uninfected parental T cells of the same donor. A number of known T-cell activation genes were isolated. Viral *stpC/tip* cDNAs were enriched after subtraction. In addition, the viral immediate-early, superantigen-homologous gene *ie14/vsag* was represented by numerous cDNA clones that comprised the entire spliced transcript. Whereas a weak basal expression of *ie14/vsag* was detected by reverse transcription-PCR only, the phorbol ester-induced transcripts were readily shown by Northern blotting. *ie14/vsag*, which before had been classified as a major immediate-early gene of herpesvirus saimiri, is localized within a highly conserved region with extensive homologies to the cellular genome. Mutant viruses without the *ie14/vsag* gene are replication competent and fully capable of transforming human and marmoset T cells. Since *ie14/vsag* is transiently expressed after stimulation, it may increase T-cell proliferation in an activation-dependent and superantigen-like but apparently Vβ-independent way.

Human T cells are transformed to stable growth in culture after infection with herpesvirus saimiri C488, a tumor virus of New World monkeys (7; reviewed in references 22 and 48). The growth-transformed cells retain many essential features of normal T cells, such as surface phenotype, early signal transduction, interleukin-2 (IL-2) dependence, antigen-specific reactivity, inducible cytotoxicity, and cytokine production (6, 7, 11, 14, 22, 48, 50, 73). The cells have a normal karyotype (72) and harbor the viral genome at a high copy number in a nonintegrated episomal form without production of virus particles (7, 23, 24). The herpesvirus saimiri transformation-associated protein StpC and the tyrosine kinase-interacting protein Tip were demonstrated in C488-transformed human T cells (9, 23, 24). Expression of other viral genes was not detected in Northern blotting experiments including purified polyadenylated RNA (23). Both proteins, StpC and Tip, are translated from a single 1.7-kb transcript, which is regulated by cellular functions and strongly expressed after T-cell stimulation (23, 24). StpC is a viral phosphoprotein with transforming capacity which interacts with cellular Ras (29, 31–34, 38, 45, 51). Tip did not exhibit transforming effects but was shown to bind specifically to the T-cell tyrosine kinase Lck and to serve as a substrate for Lck (9, 35, 36, 41–43, 74).

The genome of herpesvirus saimiri harbors a series of genes with pronounced homology to cellular genes; these herpesvirus saimiri genes are not conserved in other herpesvirus subfamilies (3). These genes are homologous to genes for members of the cytokine network such as IL-17, IL-8 receptor (IL-8R), and superantigens (1, 28, 57, 63, 75–77); to genes for complement regulation molecules such as CD59, CD46, and CD55 (2, 4, 27, 62); to genes for nucleotide metabolism enzymes such as dihydrofolate reductase (DHFR) and thymidylate synthase (TS) (10, 71); to genes for cyclins (37); and to genes for apoptosis regulation factors such as Bcl-2 and FADD (53, 67, 70). Most genes with homology to the cellular genome have been shown to code for proteins with homologous functions.

The lytic immediate-early transcription of herpesvirus saimiri A11 was studied in detail (57). Genes with immediate-early expression characteristics were localized to the *Hin*dIII-G and *Eco*RI-I/E fragments. A 52-kDa phosphoprotein was shown to be encoded by the gene in the *Eco*RI-I/E fragment. When the entire nucleotide sequence of strain A11 had been determined (3), the genes in the *Hin*dIII-G and *Eco*RI-I/E fragments were renamed open reading frame 14 (*orf14*) and *orf57*, respectively. Already in the initial description, a peptide sequence homology between *ie14* and an open reading frame in the long terminal repeats of mouse mammary tumor virus and endogenous murine retroviruses was recognized (3, 57). These open reading frames code for well-characterized viral superantigens (vSag). Recently, the function of the immediate-early superantigen homolog IE14/vSag of herpesvirus saimiri was further elucidated. The glycosylated protein is released from transfected cells and is capable of binding to major histocompatibility complex (MHC) class II HLA-DR molecules. Although a specificity for certain Vβ chains of the T-cell receptor was not described, IE14/vSag was shown to support the proliferation of human T cells (76). However, the biological role of IE14/vSag in virus replication and in T-cell transformation remained to be determined.

We applied a subtractive hybridization technique based on representational difference analysis in order to study virus gene expression with enhanced sensitivity. Stimulated virus-transformed cells were compared with noninfected parental T cells from the same donor. As expected, 12-*O*-tetradecanoyl phorbol 13-acetate (TPA)-inducible viral *stpC/tip* cDNAs were heavily enriched after subtraction. Furthermore, transcripts of

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the viral immediate-early, superantigen-homologous gene *ie14/vsag* (57, 76) were detected in abundance after T-cell stimulation. Without stimulation, a weak basal expression of *ie14/vsag* was detectable by reverse transcription-PCR (RT-PCR) only. We further show that *ie14/vsag* deletion mutants replicated as well as wild-type virus. Moreover, the *ie14/vsag* deletion viruses were fully capable of transforming human and marmoset T cells in culture. Thus, this gene is not required for replication and growth transformation. The IE14/vSag protein might be capable of activating T cells, similar to superantigens, but apparently without Vβ specificity in the human system.

MATERIALS AND METHODS

Cells and cell culture. Peripheral blood mononuclear cells (PBMC) from a healthy volunteer blood donor were isolated by density gradient centrifugation. Portions of these cells were initially stimulated with phytohemagglutinin (PHA) (5 μg/ml) (Murex/Wellcome, Grossburgwedel, Germany) and IL-2 (50 U/ml) (Proleukin; Chiron, Ratingen, Germany) and grown for 3 weeks until harvest for RNA preparation and subtraction analysis. Alternatively, the cells were infected with herpesvirus saimiri C488 and cultivated in the presence of IL-2. Six months after infection and 3 months after growth transformation was evident, the rapidly growing transformed T cells were additionally activated with TPA (2 ng/ml) (Sigma, Deisenhofen, Germany) and harvested after 4 h for RNA preparation and subtraction analysis. The resulting transformed polyclonal T-cell line 3C has been analyzed in detail (23–25, 74). CB-15 is a human CD4-positive T-cell line (7). Virus cultures, T-cell cultures, and transformation experiments were done according to published protocols (22). PHA-activated primary T cells from PBMC of *Callithrix jacchus* and *Saguinus fuscicollis* were repeatedly stimulated with irradiated (120 Gy) human feeder cells and PHA (5 μg/ml) at intervals of at least 1 month and expanded in presence of low concentrations of IL-2 (10 U/ml) in order to obtain sufficient material for parallel in vitro transformation experiments.

RNA and cDNA analysis. Total cellular RNA was prepared by the acidic phenol extraction method (12). Polyadenylated mRNA was purified with oligo(dT) Dynabeads (Dynal, Hamburg, Germany). cDNA was generated by using Moloney murine leukemia virus reverse transcriptase (Clontech, Heidelberg, Germany). The second strand was synthesized with a mix of DNA polymerase I, RNase H, *Escherichia coli* DNA ligase, and T4 DNA polymerase. Double-stranded cDNA was digested with *Rsa*I to create small fragments. Specific adapters were ligated to the cDNA fragments in order to allow subtraction based on representational difference analysis (PCR-Select; Clontech). Advantage Klen-Taq polymerase (Clontech) was applied in PCRs. Subtracted PCR products were cloned into pCR2.1 (Invitrogen, De Schelp, The Netherlands). cDNA plasmids were sequenced by using M13 reverse and T7 primers with the dye didesoxy terminator method (ABI, Weiterstadt, Germany). Northern, RT-PCR, and primer extension experiments were done by standard protocols (5). The primer extension product sizes were estimated from sequence ladders on the same gel. DNA fragments were labeled with [^{32}P]dATP by the random priming method (21). The oligonucleotides HF102 (AGT-TCA-GGC-TGT-AGC-ACA-GGC-TGC-TC) and HF103 (GGA-GCA-CCA-GAA-GAA-GGT-CGA-ATT-G) were used for primer extension experiments. *stpC* DNA was amplified with primers HF39 (GAG-TTT-CCA-AAA-TGT-ACT-AAG-CTA-AC) and HF40 (ACT-AAT-AAA-AAG-TTC-CAC-ACA-ACT-AAC). Primers HF75 (GTG-TAT-CTC-AAA-CTC-AAC) and HF76 (CTT-GTT-TGC-TAT-AAC-TTA-GTG) were used for PCR with *orf13/vil17* DNA. HF121 (GAG-CCA-AAC-ATA-GCA-TAA-TCC) and HF122 (AGA-AGG-AAC-GCA-ATT-CGA-C) were applied for genomic DNA and for RT-PCR with *ie14/vsag*. Radioactively labeled filters were exposed on BAS-III imaging plates (Fuji, Tokyo, Japan) and scanned with a BAS2000 bioimaging analyzer (Fuji). The signals were analyzed with the program TINA2.0 (Raytest, Straubenhardt, Germany).

Construction of viral deletion mutants. Virion DNA of strain C488 (8, 15) was digested with the restriction enzymes *Hin*dIII and *Xba*I in parallel reactions. The resulting overlapping fragments were cloned into the Bluescript KS+ vector (Stratagene, Heidelberg, Germany). A 3.716-kb *Xba*I fragment of herpesvirus saimiri C488 carried *ie14/vsag* at a central position (plasmid x50). The major part of the *ie14/vsag* reading frame was removed by using the C-terminal *Nco*I (nucleotide position 1470) and the N-terminal *Bst1107*I (nucleotide position 2060) sites. The simian virus 40 enhancer-driven neomycin resistance gene from pSV2neo was inserted in the antisense orientation relative to *ie14/vsag* (see Fig. 6A). Insert DNA was released from the plasmid backbone by *Not*I-*Sal*I digestion. Cleaved plasmid DNA was transfected into OMK cells (13) by calcium phosphate coprecipitation (5). On the following day, the transfected OMK cells were split into parallel flasks and infected with supernatant of herpesvirus saimiri C488 at a multiplicity of infection of about 0.5. The resulting virus-containing supernatants from lysed cultures were transferred to fresh OMK cells and passaged three times in the presence of increasing concentrations of G418 (100, 200, and 300 μg/ml in Dulbecco modified Eagle medium with 10% fetal calf serum). Virus grown in the presence of high G418 concentrations was plaque purified in methyl cellulose. The procedures largely followed published protocols (22). Plaque-purified viruses were further amplified on OMK cells in the presence of 300 μg of G418 per ml. Virus particles from 1.5 ml of supernatant were harvested by centrifugation and lysed with sodium dodecyl sulfate and proteinase K (22). The extracted DNA was digested with restriction enzymes and tested by Southern hybridization with both virus- and Neo^r-specific radiolabeled probes (21). The mutant viruses were further tested with PCRs specific for *stpC* and *ie14/vsag*. DNA from transformed cells was prepared by lysis in PCR buffer supplemented with Tween 20 and proteinase K. The proteinase was inactivated by being heated to 95°C for 10 min.

Flow cytometry. Human T cells which were transformed by wild-type or deletion mutant viruses were analyzed by flow cytometry with standard antibodies for T-cell surface epitopes on a FACStrak flow cytometer (Becton Dickinson, Heidelberg, Germany). Directly labeled antibodies, directed against CD3 (αLeu-4, SK7), CD4 (αLeu-3a, SK3), CD8 (αLeu-2a, SK1), CD45 (αHLe-1, 2D1), CD56 (αLeu-19, MY31), and CD69 (αLeu-23, L78) (all from Becton Dickinson), were applied. A broad range of monoclonal antibodies against different human Vβ epitopes (25, 59, 60) (kindly provided by Immunotech, Marseille, France, and T-Cell Sciences, Cambridge, Mass.) were applied in indirect staining reactions from parallel T-cell cultures.

CD2 hyperreactivity tests. Transformed T cells (5×10^4) were incubated alone or in the presence of antibody and/or 5×10^4 stimulator cells in 200 μl of complete RPMI 1640 medium in flat-bottom wells. The rat monoclonal antibody 39C1.5 (Immunotech), recognizing the human T11.1 epitope on the CD2 molecule, was used in stimulation and blocking assays at 1 μg/ml. The human Hodgkin's lymphoma cell line L428 was applied as a source of cell-bound CD58, which binds to CD2. The murine B-cell line A20 does not provide functional CD58 but carries large amounts of Fcγ receptors used for cross-linking the stimulatory antibody 39C1.5 (24, 49). Combined stimulation by PHA (1 μg/ml) and L428 cells served as a positive control. The supernatants were harvested after 24 h. The duoset human gamma interferon antibody pair (Genzyme, Rüsselsheim, Germany) was applied according to the manufacturer's protocol to determine gamma interferon concentrations in 1:100-diluted supernatants by enzyme-linked immunosorbent assay (ELISA). All assays were performed in triplicate. The optical densities had low standard deviations (<15%).

Nucleotide sequence accession number. The nucleotide sequence of herpesvirus saimiri C488 described in this report is available under accession no. Y13183 in the EMBL sequence data library.

RESULTS

Differential gene expression in human T cells after transformation with herpesvirus saimiri. Cellular and viral gene expression in transformed human T cells was studied by applying a PCR-mediated subtractive hybridization procedure based on representational difference analysis approaches. cDNA plasmids were recovered from TPA-activated virus-transformed human 3C T cells after subtraction of cDNAs from nontransformed T cells of the same donor. Cellular and viral activation genes were isolated (Table 1). About 400 cDNA clones were sequenced. Ninety-one cDNA clones represented known cellular genes. Twenty-eight others had not been described before. None of the cellular cDNA clones represented known nontranscribed genomic regions, which largely excludes contamination of the cDNA library by genomic DNA. Among the induced cellular genes were those for mitogen-activated protein (MAP) kinase phosphatase-2, dual-specificity protein phosphatase, early growth response protein-2 (EGR-2), and IL-13. The inducible expression of these genes was confirmed by Northern blotting (Fig. 1). Furthermore, genes for cytokines such as gamma interferon, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-13 and for surface molecules such as IL-2R gamma chain, CD48, CD97, and transferrin receptor were represented. Genes for typical housekeeping functions as well as for enzymatic reactions and signal transduction also were contained in the subtractive library; among these were genes for serine esterase, granzyme B, CREM, JunB, IkB, and calmodulin (Table 1). Only a few cDNA clones were found constitutively expressed, such as those for Rb, cyclin I, and laminin receptor (Fig. 1).

Eighty-two cDNA plasmids represented the viral *stpC/tip* transcript, which had been shown before to be expressed upon

TABLE 1. Cellular and viral activation genes isolated by subtractive hybridization

Gene represented by cDNA clones (n = 399)	Accession no.	n
Cellular genes (n = 119)		
Cytokine genes		
GM-CSF	M11734	8
Gamma interferon	A13123	6
IL-13	L06801	2
LAG-1, putative cytokine	X53683, X16066	2
LAG-2, NKG-5, lymphokine	A00142, X54101	3
Surface molecule genes		
IL-2R gamma chain	D11086	1
Transferrin receptor	X01060	1
CD48	M63911	1
CD97	X94647	1
T-cell receptor Vβ	X58805, M11956	2
MHCII-DQα	M26041	1
Enzyme and signal transduction molecule genes		
Casein kinase II b subunit	M30448	1
Serine esterase	J04071	1
Granzyme B	A26437	4
Dual-specificity protein phosphatase	U15932	5
MAP kinase phosphatase-2	U21108, U48807	1
Cyclin I	D50310	1
CREM	D14826	1
EGR-2	J04076	2
JunB	X51345	1
Mitogen-induced nuclear orphan receptor	U12767	1
IkB/MAD-3	M69043	2
Hsp86	X07270	1
Elongation factor 1α subunit	X03558	1
Calmodulin 1	U16850	2
Rb	M28419	1
Genes with probable housekeeping functions		24
Genes without known function		7
Genes with equivalents in the murine genome		7
Unknown genes		28
Viral genes (n = 280)		
stpC/tip	M55264	82
orf14/ie14/vsag	X64346	186
orf57/ie57	X64346	10
orf70/ts	X64346	1
orf74/vIL-8R (ECRF3)	X64346	1

activation (23, 24). The isolated *stpC/tip* cDNAs started at position 1875 (according to the sequence under GenBank accession no. M55264) (8), shortly after the main inducible transcription start (23). The cDNAs comprised the whole unspliced transcript down to poly(A) sequences starting at position 399 or 409 in the terminal L/H-DNA transition (accession no. M55264) (8). Surprisingly, the viral immediate-early, superantigen-homologous gene *ie14/vsag* was represented by numerous cDNAs, which again covered the whole mRNA and showed splicing upstream of the reading frame (Fig. 2A). The transcription start was further mapped by primer extension experiments, using two different primers, to position 28942 according to the A11 genome (3) (GenBank accession no. X64346) (Fig. 2B). The size difference between the extension products results from the distance between the primers. Primer HF102 (A11 positions 28833 to 28853) produced a band of 110 bp, and primer HF103 (A11 positions 28863 to 28887) generated an 80-bp product. In addition to the numerous *ie14/vsag* plasmids, 10 cDNA clones were found for the viral immediate-early gene *ie57*, comprising almost the whole spliced transcript (Fig. 3A), and a single cDNA fragment was found for the viral TS gene. In both cases, a very weak hybridization signal was observed at the expected position by Northern hybridization of RNA from TPA-induced cells (Fig. 3B). Whereas an overnight exposure was sufficient to visualize *ie14/vsag* signals after hybridization of Northern blot filters, the *ie57* and TS gene signals required prolonged exposures of the filters to imager screens, for up to 10 days. Another single cDNA clone displayed the sequence of the viral IL-8R (ECRF-3). However, in this case, even Northern hybridizations with isolated mRNA from TPA-stimulated transformed T cells remained negative repeatedly. As there are no indications of splicing in the *orf74/il-8r* area, RT-PCR experiments cannot be considered relevant, due to the high-copy-number persistence of episomal viral genomes in the transformed T cells. Thus, the viral genes expressed in transformed T cells after stimulation are *ie57* and the TS gene at low intensity and *ie14/vsag* in abundance, in addition to the known transformation-associated gene *stpC/tip*.

The superantigen-homologous gene *ie14/vsag* is transiently transcribed after stimulation of herpesvirus saimiri-transformed T cells. The viral gene *ie14/vsag* was abundantly expressed in TPA-stimulated virus-transformed human T cells, as shown by Northern hybridization. The induction was transient after stimulation. Signals were first seen after 2 h, reached maximal values between 4 and 8 h, and returned to negative

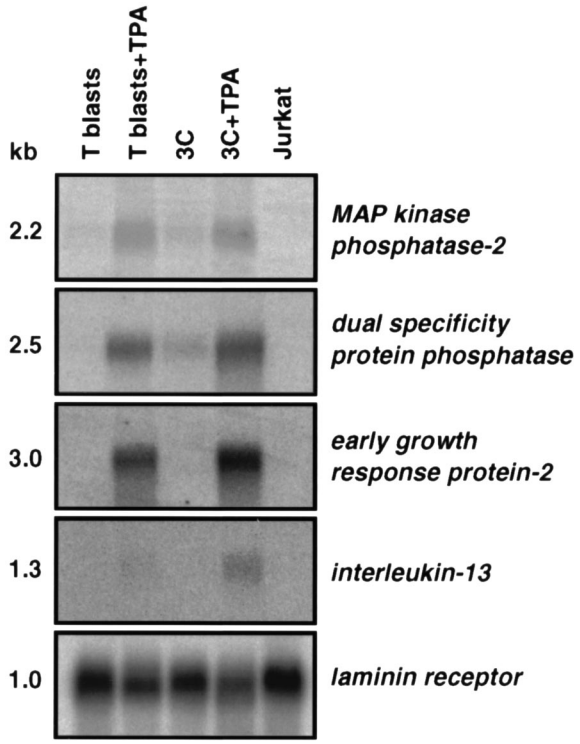

FIG. 1. Activation-induced T-cell gene expression. Total cellular RNAs from PHA-activated T blasts and herpesvirus saimiri-transformed T cells (3C) (23, 24, 25, 74) with or without TPA stimulation, as well as RNA from the human leukemia cell line Jurkat (66), were transferred onto nylon filters and probed with various cDNA fragments (Table 1) from the subtracted cDNA library. As an example, the expression patterns are shown for the MAP kinase phosphatase-2, dual-specificity protein phosphatase, EGR-2, and IL-13 genes. All these genes show induction after 6 hours of T-cell stimulation by TPA in both nontransformed and transformed T cells. In contrast, laminin receptor transcription is not affected by T-cell stimulation. The transcript sizes are given in the left margin.

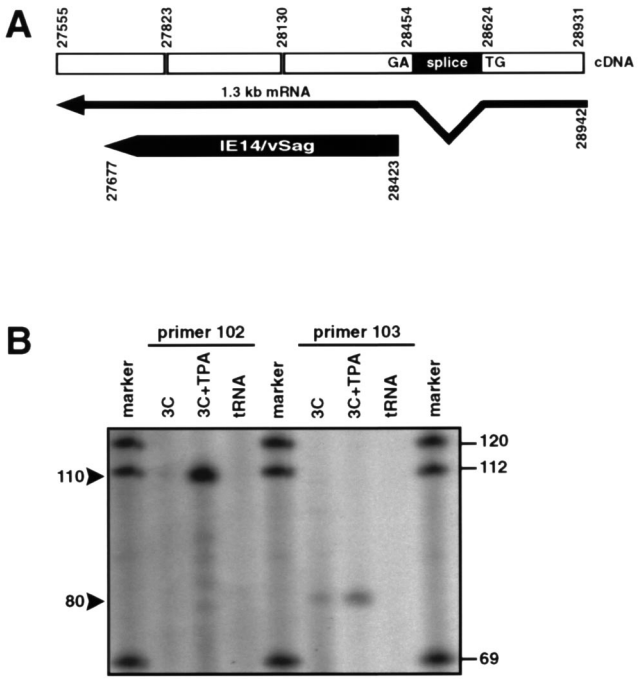

FIG. 2. Gene structure and transcription initiation of *ie14/vsag*. (A) Numerous cDNA clones (n = 186) representing *ie14/vsag* were isolated from the subtracted cDNA library. The sequences of these clones were aligned. In front of the translation start, an intron region was identified between positions 28624 and 28454 according to the sequence of the A11 prototype genome (3) (accession no. X64346). The terminal cDNA clones included poly(A) stretches starting at A11 position 27555. (B) The transcription initiation of *ie14/vsag* was determined by primer extension experiments with RNA of stimulated transformed T cells by using two oligonucleotides, HF102 and HF103. Product sizes were estimated from sequencing reactions on the same gel. The size difference between the observed bands (110 versus 80 bp) reflects the distance between the primers on the genomic DNA. Both signals correspond to a transcription start at A11 position 28942, shortly upstream of the first cDNA clones. Numbers on the left and right represent base pairs.

after 24 h (Fig. 4A). In contrast to the case for *stpC/tip*, transcription of *ie14/vsag* is not stimulated by cycloheximide treatment (data not shown). When RT-PCR was performed with *ie14/vsag*-specific primers flanking the intron, a weak basal expression signal was observed in unstimulated cells (Fig. 4B). In contrast, induced cells had large amounts of spliced *ie14/vsag* transcripts. In the lanes for noninduced cells, another band representing the genomic variant was prominent, which was only faint in stimulated cells. This probably reflects primer competition and abundance of the spliced transcript after stimulation (Fig. 4B). The pattern of *ie14/vsag* gene expression was similar in human αβ or γδ T cells or macaque T cells. Moreover, there was no difference between C488- and C139-transformed human T cells. In contrast, in the nonproductive marmoset T-cell lines 1670, L77/5, H1591, and 70N2, which were derived mostly from tumors (20, 56, 65, 69), this gene was not inducible (data not shown).

***ie14/vsag* is localized in a highly conserved genomic region of herpesvirus saimiri.** Overlapping genomic DNA clones from viral *Xba*I and *Hin*dIII libraries, comprising the region between *orf12* and *orf25* (16,552 nucleotides; accession no. Y13183) (Fig. 5; Table 2), were sequenced. *orf12* and *orf25* were only partially cloned and sequenced. As already suggested by hybridization experiments, virus strain C488 showed high homology to the respective region of strain A11 (accession no. X64346) (3). The reading frames *orf12* to *orf25* displayed nearly perfect identity of 85.7 to 99.4% on the amino acid level and of 88.8 to 99.0% on the nucleotide level (Table 2). Beside some variations in noncoding regions, the amino acid divergences in protein 12 (14.3%) and protein 22 (glycoprotein H [gH]) (11.9%) were most evident. When the N-terminal region of gH was analyzed separately, a higher degree of variation was observed (22.3% identity within the first 750 nucleotides, or 26.4% identity within the N-terminal 250 amino acids). The proteins with homology to cellular polypeptides (vIL17/orf13, vSag/IE14, vCD59/orf15, and vBcl-2/orf16) did not show relevant sequence divergence (Table 2).

***ie14/vsag* is dispensable for virus replication and for T-cell transformation.** The major part of the *ie14/vsag* reading frame was removed from the plasmid x50 and replaced by the neomycin resistance gene in the antisense orientation (Fig. 6A). The recombination plasmid was transfected into OMK cells. After infection with wild-type herpesvirus saimiri C488, neo-

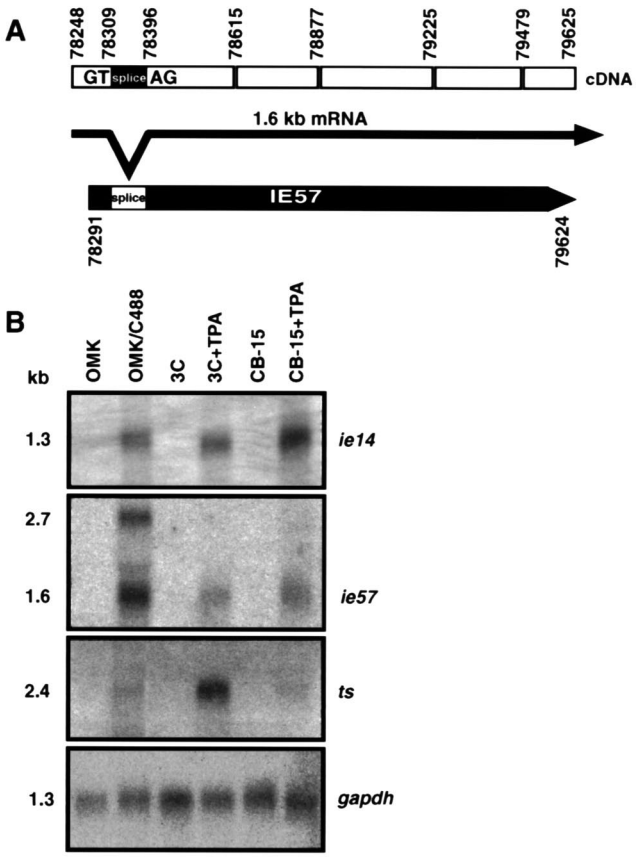

FIG. 3. *ie57* gene structure and expression of *ie14*, *ie57*, and the TS gene. (A) Ten cDNA clones from the subtractive library represented almost the entire viral immediate-early gene *ie57*. The nucleotide sequence from C488 is more than 95% identical to its A11 counterpart; the protein sequences are absolutely identical. In the N-terminal portion of the translated region, an intron between A11 positions 78309 and 78396 was identified. The cDNAs stopped at A11 position 79625, just at the end of the open reading frame. (B) The expression levels of *ie14/vsag*, *ie57*, and the TS gene (*orf70*) were compared by rehybridizing Northern filters with RNAs from noninfected and C488-infected OMK cells and from herpesvirus-transformed 3C and CB-15 T cells with or without TPA stimulation. Signals were observed under lytic conditions and after TPA stimulation of transformed human T cells. Whereas a strong signal was observed for *ie14/vsag*, weak bands were detected for *ie57* and the TS gene. Exposure times were about 10 days for *ie57* and the TS gene, whereas 10 h was sufficient for *ie14/vsag*. Under lytic conditions, *ie57* shows transcripts of 2.7 and 1.6 kb. Transcript sizes are given in the left margin. The glyceraldehyde phosphate dehydrogenase gene (*gapdh*) served as positive internal control.

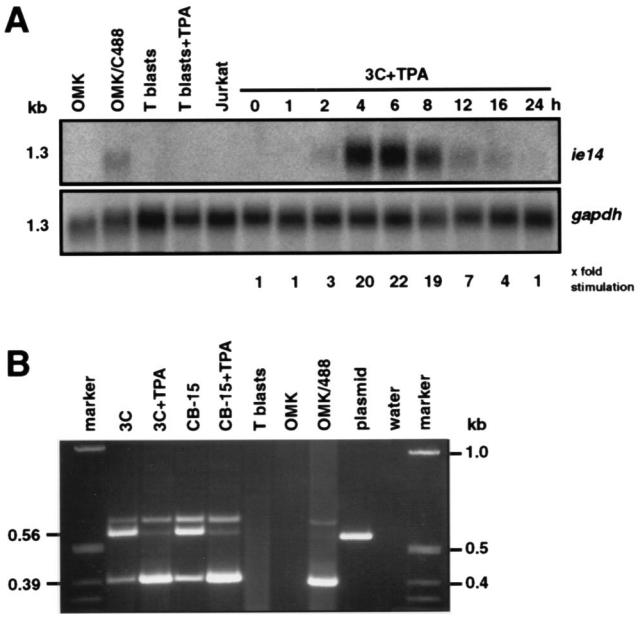

FIG. 4. Inducible expression of *ie14/vsag*. (A) Total cellular RNAs from OMK cells with or without infection by herpesvirus saimiri C488, from primary T cells with or without stimulation, from Jurkat tumor cells (66), and from herpesvirus-transformed 3C cells at various times after TPA stimulation were hybridized with DNA probes specific for *ie14/vsag* and for the glyceraldehyde phosphate dehydrogenase gene (*gapdh*). *ie14/vsag* was found to be expressed during lytic replication in OMK cells and after T-cell stimulation. The photostimulated luminescence counts determined for *ie14/vsag* during the time course were normalized for the endogenous *gapdh* expression. The relative values are given as fold stimulation. After stimulation, strong *ie14/vsag* signals were observed at 4 to 8 h only. (B) RNAs from stimulated and unstimulated herpesvirus saimiri-transformed 3C and CB-15 cells were subjected to *ie14/vsag*-specific RT-PCR in parallel with RNA controls from PHA-activated T lymphoblasts and noninfected and infected OMK cells, as well as with a plasmid DNA control (x50). Strong signals for the spliced transcript were detected at 390 bp after TPA stimulation of herpesvirus-transformed T cells. Without stimulation, these signals were much weaker. A band of 560 bp represents genomic viral DNA. Due to primer competition, this band is very weak after stimulation or during lytic infection. An additional band of about 600 bp is most probably an artifact.

mycin-resistant virus populations were enriched, which resulted from recombination processes. Finally, plaque-purified virus clones were obtained from two independent experiments and analyzed by Southern hybridization (Fig. 6B), which showed the expected band sizes with transgene- and backbone-specific radioactive DNA probes. The mutant viruses were further tested by PCRs for *stpC* and *ie14/vsag* (Fig. 6C). The mutants showed signals for *stpC* but did not exhibit bands for *ie14/vsag*, in contrast to wild-type virus C488. The virus mutant 14-3.1 was found to lack the *stpC* band, in addition to having the expected *ie14/vsag* deletion. By Southern blotting, the additional deletion was mapped to the whole transformation-related region, including *stpC*, *tip*, and their regulatory sequences. *orf2* (encoding DHFR) and *orf3* (encoding a structural protein) remained unaffected. The *ie14/vsag* virus mutants 14-3.10, 14-4.5, and 14-4.6 were selected for subsequent experiments. 14-3.1 served as an *stpC/tip* deficient control. The described deletions did not affect viral replication: the viruses were cloned and grown to high titers ($>10^7$ PFU/ml).

T cells from humans and from different marmoset monkey species were transformed to stable growth in culture after infection by wild-type virus or by the *ie14/vsag* deletion mutants, with the exception of mutant 14-3.1, which additionally lacks the *stpC/tip* gene (Table 3). The cultures were stably proliferating over observation periods of 6 to 12 months; no difference was discernible between cells transformed by wild-type C488 or by the *ie14/vsag* single-knockout mutants. The retained deletion in the viral genome was confirmed by PCR of total cellular lysates of each of the transformed cultures (Fig. 7). The surface phenotypes of human T cells transformed by wild-type virus or the *ie14/vsag* mutants were also highly similar. The cells carried CD4 or CD8, and CD3, CD45, CD56, and CD69, with similar intensities (data not shown) irrespective of whether wild-type virus or *ie14/vsag* mutants had been used for transformation. There was also no difference between the mutant viruses from independent experiments (e.g., 14-3.10 versus 14-4.6). The complete series of cell lines resulting from the experiment with donor 120-CB (Table 3) was tested for CD2 hyperreactivity, according to previous observations that in contrast to nontransformed cells, C488-transformed T cells react with cytokine production and proliferation to stimulation of a single CD2 epitope (T11.1) by CD2-specific antibodies or CD58 (24, 49, 73). The inducible production of gamma interferon was measured by ELISA (Table 4). Analogous results were obtained by tumor necrosis factor alpha ELISA (data not shown). CD58-bearing L428 cells stimulated cytokine production. This stimulation was efficiently blocked by addition of antibodies to CD2. The complementary assay used murine A20 cells lacking functional CD58. CD2 antibody cross-linked by Fcγ receptors on A20 cells efficiently stimulated gamma interferon production. The CD2 hyperreactivities of all cell lines tested were nearly identical, irrespective of the mutation in the virus genome. Thus, *ie14/vsag* is dispensable for replication and T-cell transformation in culture.

Herpesvirus saimiri C488 transforms human T cells without Vβ preference. In order to further investigate a potential superantigenic function in transformation, we analyzed T-cell receptor Vβ expression by flow cytometry with about 60 different Vβ-specific antibodies (60) during parallel transformation experiments with the same human PBMC preparation. Table 5 shows data for only a few monoclonal antibodies, for which significant changes were observed. During the first weeks, the populations were polyclonal without overt differences and without enrichments for specific Vβ chains. After 4 months, an enrichment of Vβ6.7-positive T cells to 55.7% was observed in culture C. This enrichment was unstable, as the Vβ6.7-positive cells had decreased again to 0.8% after 9 months. However, after 9 months in culture, there was evi-

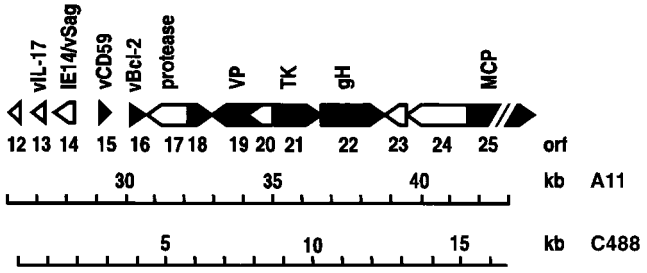

FIG. 5. Genomic organization of the *orf12*-to-*orf25* region in C488. The genomic sequence of a region of 16,552 nucleotides from virus strain C488 (accession no. Y13183) was determined from libraries of *Hin*dIII and *Xba*I clones in Bluescript KS(+). The sequence is highly similar to that of prototype strain A11 (3) (accession no. X64346) (Table 2). *orf12* and *orf25* have been cloned and sequenced only partially. The position of the C488 sequence relative to the A11 genome is indicated. The genes *vil-17*, *ie14/vsag*, *vCD59*, and *vbcl-2*, which are homologous to cellular genes, do not show relevant sequence variation among virus strains that are able (C488) or unable (A11) to transform human T cells in culture. MCP, major capsid protein.

TABLE 2. Sequence conservation between herpesvirus saimiri strains C488 and A11

Nucleotide sequence (*orf*)[a]	Identity (%)	Nucleotides (*n*)	Protein sequence[b]	Identity (%)	Similarity (%)	Amino acids (*n*)
12	91.9	210	12	85.7	85.7	70
Noncoding *12–13*	90.6	392				
13	97.4	453	13 (vIL-17)	98.0	98.7	151
Noncoding *13–14*	81.5	205				
14	96.4	747	14 (IE14/vSag)	95.1	95.6	248
Noncoding *14–15*	93.3	804				
15	93.1	348	15 (vCD59)	91.4	92.2	116
Noncoding *15–16*	92.9	747				
16	94.6	480	16 (vBcl-2)	92.5	95.6	160
Noncoding *16–17*	81.6	38				
17	96.5	1,425	17 (protease)	96.2	96.4	474
18	96.4	768	18	97.3	97.7	256
19	97.7	1,629	19 (VP)	97.8	98.3	543
20	97.2	909	20	95.7	95.7	303
21	96.0	1,581	21 (TK)	96.0	96.8	527
22	88.8	2,151	22 (gH)	88.1	91.2	717
23	98.2	758	23	98.8	99.2	253
Noncoding *23–24*	100.0	12				
24	97.9	2,187	24	98.1	98.4	729
Noncoding *24–25*	100.0	10				
25	99.0	1,017	25 (MCP)	99.4	99.7	339

[a] *orf12* and *orf25* have not been cloned and sequenced entirely.
[b] TK, thymidine kinase; MCP, major capsid protein.

dence that pure, but different, T-cell clones were present in the four parallel cultures (Table 5). At that time, culture A displayed Vβ5.2/3, culture C displayed Vβ8.1/2, and culture D displayed Vβ5.1, exclusively. In culture B, none of the Vβ antigens tested was represented above 1%. As the panel of 60 monoclonal antibodies does not yet cover the whole Vβ spectrum, culture B probably carries one or a few Vβ chains that are not recognized by the antibody panel. Most certainly, the results at 9 months reflect clonal overgrowth. Thus, the Vβ phenotype of the transformed T cells seems not to be determined by the viral superantigen.

DISCUSSION

Cellular gene expression in herpesvirus-transformed human T cells. The representational difference analysis method was used to search for cellular and viral genes that specify stimulated T cells transformed by herpesvirus saimiri C488. Earlier immunological analyses revealed multiple similarities between primary activated T lymphocytes and herpesvirus-transformed T cells (7, 11; reviewed in references 22 and 48). Only a few differences were observed: C488 transformed T cells are hyperreactive to CD2 ligation (24, 49), they aberrantly express the tyrosine kinase Lyn as a functional enzyme (24, 74), and they can be stimulated to produce extraordinarily high amounts of gamma interferon (11, 24, 73). Correspondingly, most of the cellular cDNAs isolated in this study are also typical for normal activated T cells; these include cDNAs for gamma interferon, GM-CSF, and IL-13. The surface markers IL-2R gamma chain, transferrin receptor, CD48, CD97, and MHC class II molecules are known to be expressed by highly activated T cells, as are serine esterase, granzyme B, CREM, EGR-2, and JunB (Table 1). The induced transcription of protein phosphatases may play a role in feedback regulation after stimulation. Whereas expressions of MAP kinase phosphatase-2 and dual-specificity protein phosphatase were identical in nontransformed and transformed T cells after activation, the transcript levels of EGR-2 and IL-13 were higher in transformed T cells (Fig. 1). Further studies are required to

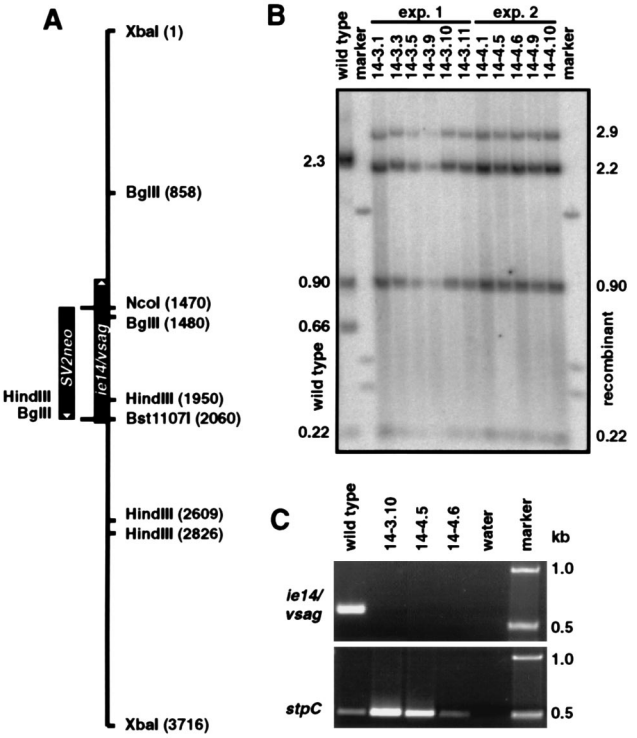

FIG. 6. *ie14/vsag* deletion mutants. (A) In order to construct a recombination plasmid, the major part of the coding sequence for IE14/vSag was cut out from plasmid x50 by using the restriction enzymes *Nco*I and *Bst*1107I. Into this deletion, the neomycin resistance gene from pSV2neo was inserted in the antisense orientation. The relevant restriction enzyme sites are given at their positions in plasmid x50 (accession no. Y13183). (B) *Hin*dIII-digested viral DNAs from the wild type and from selected *ie14/vsag* deletion mutants from two independent experiments were subjected to Southern blot hybridization with the radioactive labeled insert from plasmid x50. The expected fragment sizes are shown on the left for the wild type and on the right for the recombinants. Expected and observed band patterns were identical. (C) The presence of the deletion in the recombinant viruses was confirmed by DNA PCR for *ie14/vsag*. In contrast, the *stpC/tip* gene was not affected.

TABLE 3. In vitro transformation of human and marmoset T cells with wild-type and recombinant viruses[a]

Donor	Species	Mo	Virus genotype				
			C-488 (wt)	14-3.1 (Δ14 Δ*stpC*/*tip*)	14-3.10 (Δ14)	14-4.5 (Δ14)	14-4.6 (Δ14)
5772774	Human	12	4/4	0/4	ND	ND	3/4
120-CB	Human	6	2/2	0/2	2/2	ND	2/2
RA-CB	Human	6	2/2	0/2	2/2	ND	2/2
SU	*C. jacchus*	6	1/1	0/1	2/2	1/1	2/2
87	*S. fuscicollis*	6	2/2	0/2	2/2	2/2	2/2
89	*S. fuscicollis*	6	2/2	0/2	2/2	2/2	2/2
Total			13/13	0/13	10/10	5/5	13/14

[a] wt, wild type; ND, not done; CB, cord blood.

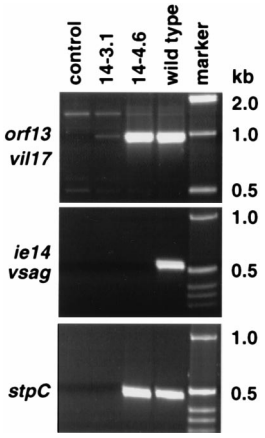

FIG. 7. Presence of the *ie14/vsag* deletion in transformed T cells. Total cellular DNA from all transformed T-cell cultures (Table 3) was analyzed by viral DNA PCR after several months of culture. An example is shown for cultures from the experiments with human 5772774 cells. In this case, PCR was performed at 1 month after infection, when the control cells still persisted without replication and the transformed cells were already proliferating quickly. Wild-type-transformed T cells display all three genes tested. In 14.4-6 transformed T cells, the signal for *ie14/vsag* is missing, whereas *stpC* and *orf13/vil-17* are not affected. In the nonreplicating cells infected by the double-knockout virus 14.3-1 for *stpC/tip* and *ie14/vsag*, a faint signal for *orf13/vil-17* is present, whereas the others are missing.

understand the roles of the genes which are unknown in function ($n = 7$) or not yet represented in the gene libraries ($n = 28$).

Rhadinovirus genes with homology to the cellular genome. Rhadinoviruses like herpesvirus saimiri and human herpesvirus 8 contain an unusually high number of genes that seem to originate from the cellular genome and are not common in other herpesvirus subfamilies (3, 55). Although the genomic structures are nearly identical in the two viruses, there are four regions where different genes have been incorporated. One of these regions with genetic divergence is situated between *orf11* and *orf17*. In this region, human herpesvirus 8 contains viral gene versions of the IL-6, MIP-1α, and MIP-1β genes, as well as the DHFR and TS genes, which, in contrast, are terminally located in herpesvirus saimiri. *vbcl-2/orf16* occurs at similar positions in the two viruses (54, 55, 64). Neither *orf13/vil-17* nor *ie14/vsag* is represented in human herpesvirus 8, and both genes are correlated with T-cell-specific effects (28, 63, 75–77). In contrast, the presence of *vil-6* in human herpesvirus 8 is suggestive for its implication in herpesvirus B-cell transformation (54). Similar to the case for herpesvirus saimiri-transformed T cells, only a few genes of human herpesvirus 8 seem to be expressed in transformed human B cells and in most tumor cells of Kaposi's sarcomas (23, 79). The distantly related herpesvirus saimiri strains A11 and C488 are very similar throughout the whole analyzed genomic region (Table 2; Fig. 5). The genes *orf13/vil-17*, *ie14/vsag*, *orf15/vCD59*, and *orf16/vbcl-2* are more than 91% identical among viruses that are able (C488) or not able (A11) to transform human T cells. This observation raises the question of whether such genes contribute to herpesvirus T-cell transformation or whether they support replication, virus spread, and persistence in the natural host.

Herpesvirus superantigen in transformed T cells? Surprisingly, we isolated numerous *ie14/vsag* cDNA clones from the subtractive library (Table 1; Fig. 2). This gene is inducibly expressed in transformed human T cells after TPA stimulation. Faint hybridization signals were first discernible at 2 h, and strong signals were observed at 4 to 8 h, after stimulation (Fig. 4). In contrast, the transformation-associated *stpC/tip* gene is already activated at less than 1 h after activation (23). Inducible *ie14/vsag* RNA signals were seen in transformed αβ and γδ cells (24, 39, 58, 74). *ie14/vsag* transcript levels were also inducible in C139-transformed T cells, in which the *stpC/tip* message does not react on TPA stimulation (24). In contrast, the *ie14/vsag* gene was not inducible in long-term-cultivated T-cell lines derived mostly from herpesvirus saimiri-induced marmoset tumors (20, 56, 65, 69). Whereas *stpC/tip* transcript levels were also stimulated by cycloheximide (23), *ie14/vsag* was not induced in the absence of protein synthesis. Although *ie14/vsag* responds to T-cell activation, it would thus not be classified as a classical early response gene (30, 80).

We further investigated the functional role of *ie14/vsag* during transformation of human T cells. However, no enrichment of specific Vβ family members could be observed during the transformation process, which ends in clonal selection of the fastest-growing cells (Table 5). This is compatible with results obtained by M. Spriggs (Seattle, Wash.), who observed a non-Vβ-specific proliferation of primary T cells after addition of recombinant IE14/vSag protein (68). As superantigens are known to display some species specificity, such a superantigenic function might be relevant especially in the natural host of the virus, the squirrel monkey *Saimiri sciureus.*

Herpesvirus saimiri-transformed human T cells do not reactivate the virus after TPA treatment (23). TPA stimulation is, however, an efficient way to induce Epstein-Barr virus replication in transformed B cells (81). Similarly, induction of uncharacterized viral antigens by chemical stimulation has been described for herpesvirus saimiri-transformed monkey cells, but without induction of virus particle production (56). Such viral antigens might be identical to the products of the genes de-

TABLE 4. CD2 hyperreactivity of transformed human T cells resulting in gamma interferon production

Cell line[a]/addition	Gamma interferon production (ng/ml) by cell line:					
	C488 A	C488 B	14-3.10 A	14-3.10 B	14-4.6 A	14-4.6 B
Medium	1.84	2.01	4.05	4.47	2.45	8.83
L428/PHA	37.54	43.24	65.92	59.28	63.83	70.65
L428	25.60	28.50	37.05	33.82	27.30	50.84
L428/anti-CD2	4.91	2.42	2.37	3.67	2.30	5.89
A20	3.00	1.71	2.40	2.16	2.13	3.39
A20/anti-CD2	16.09	12.37	17.51	16.84	17.32	29.43

[a] The cell lines were from the experiment with donor 120-CB (Table 3).

TABLE 5. T-cell receptor Vβ representation in parallel experiments from the same PBMC preparation

Culture	Mo	% of cells with the following Vβ chain[a]:							
		2.2 (E2.2E7.2)	5.1 (LC4)	5.1 (IMMU157)	5.2/3 (42/1C1)	6.7 (OT145)	8.1/2 (16G8)	14.1 (CAS1.1.4)	21.3 (IG125)
A	0	6.1	3.4	0.1	3.6	2.1	3.6	5.1	2.9
	1	8.0	6.7	0.0	3.0	2.9	4.1	5.2	2.5
	2	5.5	16.5	0.0	2.4	3.6	2.3	4.2	1.0
	4	5.0	19.5	1.0	4.6	1.6	1.3	1.1	0.9
	9	0.1	0.0	0.1	**98.9**[b]	0.0	0.0	0.1	0.1
B	0	6.1	3.4	0.1	3.6	2.1	3.6	5.1	2.9
	1	10.9	1.6	0.0	4.8	2.8	4.7	5.6	2.1
	2	9.1	0.5	0.0	1.0	0.5	1.2	1.5	0.6
	4	0.8	0.2	0.3	0.1	0.1	0.2	0.1	0.2
	9	0.3	0.2	0.3	0.1	0.1	0.1	0.2	0.2
C	0	6.1	3.4	0.1	3.6	2.1	3.6	5.1	2.9
	4	13.0	0.5	1.0	1.0	**55.7**	18.1	1.3	1.8
	9	0.3	0.0	0.0	0.0	0.8	**97.0**	0.1	0.1
D	0	6.1	3.4	0.1	3.6	2.1	3.6	5.1	2.9
	4	2.2	0.9	0.3	0.5	0.5	0.3	6.8	7.8
	9	0.8	0.6	**87.1**	0.2	0.3	0.2	0.2	0.2

[a] The monoclonal antibody used is given in parentheses.
[b] Boldface indicates significant enrichment.

scribed in this study. It should be kept in mind that the expression of *ie57* and of the TS gene had very low intensity. The biological significance of this observation remains unknown.

***ie14/vsag* is dispensable for lytic replication and T-cell transformation.** Herpesvirus saimiri is not pathogenic in squirrel monkeys, its natural host, but causes fulminant T-cell leukemias in other New World primates, such as *Saguinus* species (reviewed in reference 26). Functional studies, including mutagenesis of virus genomes, have focused on the transforming functions. In prototype strain A11, the transformation-associated region was mapped to a terminal genomic region of the coding L-DNA. The responsible gene was termed *stpA* (for saimiri transformation-associated protein of subgroup A). Virus mutants without *stpA* were unable to transform marmoset T cells and were not pathogenic in animal experiments (16–18, 40, 52). In viruses of subgroup C (46, 47), the transforming protein StpC and the Lck interaction partner Tip are encoded at the equivalent position (8, 9, 29, 31–34, 38, 51). In the case of strain C484, the transformation-associated region (9.2 kb), including *stpC/tip*, was transferred to a nontransforming strain, which thereby gained oncogenicity in rabbit experiments (47). Moreover, these genes were essential for short-term transformation of human T cells (44). In this study, we observed that the additional deletion of the complete *stpC/tip* gene of C488 in the context of *ie14/vsag* deletion mutants abolished transforming capacity for marmoset and human T cells (Table 3). However, the *stpC/tip* gene of C488 has to be studied independently from other mutations (19).

As only few viral genes were found to be expressed in transformed human T cells, we analyzed the contribution of the abundantly transcribed gene *ie14/vsag* to viral replication and T-cell transformation in cell culture. We eliminated the gene by homologous recombination and isolated recombinant virus clones. In order to exclude influences from accidental mutations anywhere in the large herpesvirus genome, we tested recombinant virus clones from two independent experiments (Table 3; Fig. 6 and 7). Although *ie14/vsag* had been classified as one of the two major immediate-early genes of herpesvirus saimiri (57), the *ie14/vsag* deletion viruses were as replication competent as the wild type. Moreover, the deletion mutants were fully capable of transforming human or marmoset T cells. The surface phenotype and the CD2 reactivity of transformed T cells were identical in presence or absence of *ie14/vsag* in the viral genome. The situation seems similar to that for herpes simplex virus, where only two of five immediate-early genes are essential (61). It seems possible that this gene may be coincidentially expressed under immediate-early conditions during lytic infection in vitro. We favor the hypothesis that *ie14/vsag* may play a role as a T-cell activator in leukemogenesis in marmoset monkeys or during apathogenic persistence in the squirrel monkey, the only known natural host of this virus.

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