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A transcription enhancer in the *Herpesvirus saimiri* genome

Sabine Schirm, Frank Weber[1], Walter Schaffner[1] and Bernhard Fleckenstein

Institut für Klinische Virologie der Universität Erlangen-Nürnberg, Loschgestr. 7, D-8520 Erlangen, FRG and [1]Institut für Molekularbiologie II der Universität Zürich, Hönggerberg, CH-8093 Zürich, Switzerland

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***Herpesvirus saimiri*, an oncogenic agent of New World primates, has a linear double-stranded DNA genome of ~155 kb. To test its genome for the presence of a transcription enhancer, we have mixed randomly fragmented *H. saimiri* DNA with non-infectious, linear SV40 DNA lacking the 72-bp repeat enhancer region (the so-called SV40 enhancer trap) and co-transfected this DNA mixture into monkey CV-1 cells. Viable SV40-like viruses were generated by intracellular ligation/repair processes with short *H. saimiri* DNA fragments. One recombinant, SVHS-2, had integrated a 377-bp enhancer segment from the righthand region of the *H. saimiri* genome, 7 kb upstream of DNA sequences encoding an immediate-early mRNA. This enhancer sequence is contained within the non-repetitive portions of the viral genome known to be preserved episomally in all lymphoid tumor cell lines. Further recombinant viruses (SVHS-14, SVHS-7, and SVHS-8) essentially contain subsets of the 377-bp insert. Unlike in the previous enhancer trap experiments, where heterologous enhancers were incorporated without any sequence alterations, SVHS-14 and SVHS-7 have suffered short internal deletions of a very similar segment of the *H. saimiri* insert. This renders the enhancer more active, implying that the deleted segment, while it may have a role in the herpesvirus infection cycle, exerts a negative effect within the isolated enhancer.**

Key words: enhancer trap/*Herpesvirus saimiri*/immediate early gene/transcription enhancer

Introduction

Herpesvirus saimiri is a highly oncogenic virus which causes malignant lymphomas and acute lymphocytic leukemia in numerous species of New World primates, notably marmoset monkeys, and in rabbits (for a review, see Fleckenstein and Desrosiers, 1982; Desrosiers and Fleckenstein, 1983). *H. saimiri* virion DNA, designated as M-genome, is a linear duplex molecule of ~155 kb; it consists of a long unique segment of L-DNA (112 kb, 36% G+C) which is flanked by repetitive H-DNA (70.8% G+C) of variable multiplicity (Bornkamm *et al.*, 1976). In transformed lymphoid primate cells, viral DNA persists as non-integrated, covalently closed molecules. A considerable part of viral genetic information is not required for persistence of viral DNA and/or maintenance of the transformed phenotype, as large deletions can occur in the L-DNA of the circular viral genomes (Werner *et al.*, 1977; Desrosiers, 1981; Kaschka-Dierich *et al.*, 1982; Schirm *et al.*, 1984). In recent years, transcriptional enhancers have been identified in a number of viral and cellular genomes (for reviews, see Picard, 1985; Gluzman, 1985). These DNA sequences are able to activate transcription of a linked gene in either orientation, over long distances and even from a position downstream of the cap site (Banerji *et al.*, 1981; Moreau *et al.*, 1981). The SV40 enhancer is essential for efficient expression of the early viral genes and deletion of this element renders the virus non-viable (Benoist and Chambon, 1981; Gruss *et al.*, 1981). However, this deficiency can be overcome by substituting a heterologous viral enhancer, which results in considerable T-antigen gene expression (Laimins *et al.*, 1982; de Villiers *et al.*, 1982) and even in limited virus replication (Levinson *et al.*, 1982).

We have used an efficient selection system for enhancing DNA segments, the so-called SV40 enhancer trap (Weber *et al.*, 1984) to detect DNA sequences that may be functionally relevant for transcription and replication of *H. saimiri* in lytically infected and transformed cells. This report shows that the SV40 'enhancer trap' can rescue an enhancer from the entire herpesvirus genome. The *H. saimiri* segment is located 7 kb upstream of an immediate-early coding region of the virus and is present not only in all lytically growing virus particles but also in the severely truncated viral genomes persisting in transformed cells. Unlike any other of the various enhancers selected with the 'enhancer trap' (Weber *et al.*, 1984; Boshart *et al.*, 1985; Weber and Schaffner, 1985; Dorsch-Häsler *et al.*, 1985; E.Serfling, A.Lübbe, K.Dorsch-Häsler and W.Schaffner, in preparation) two SV40-*H. saimiri* recombinants have been isolated which have suffered a very similar deletion within the enhancer. By this, the enhancer activity apparently is improved, suggesting that the deleted segments exert negative effects within the recombinants.

Results

Detection of a functional enhancer in H. saimiri DNA

Initial attempts to isolate segments of *H. saimiri* DNA having enhancer activity were done by mixing randomly fragmented complete viral M-genomes with the enhancerless linearized SV40 genome, and transfection of the DNA mixture into cells permissive for SV40 growth. The physical map of the enhancer trap with regard to transcriptional regulatory elements, cap sites and origin of DNA replication is shown in Figure 1. For a typical experiment, purified *H. saimiri* M-DNA was fragmented by sonication to fragments of 200–400 bp; the *H. saimiri* DNA was mixed with SV40 enhancer trap molecules, co-precipitated with calcium phosphate, and transfected into CV-1 (*Cercopithecus* kidney) cells. After 1 month, the CV-1 cells lysed due to growth of SV40-type viruses. Viral DNA was extracted from the infected cells and cloned into the bacterial plasmid pBR327. One of these clones, designated SVHS-2, contained an infectious SV40-*H. saimiri* recombinant virus and was analysed in more detail.

Physical mapping and nucleotide sequencing of the enhancer element in L-DNA

By restriction mapping and Southern blotting, the *H. saimiri* insert in clone SVHS-2 was narrowed to a segment of virion L-

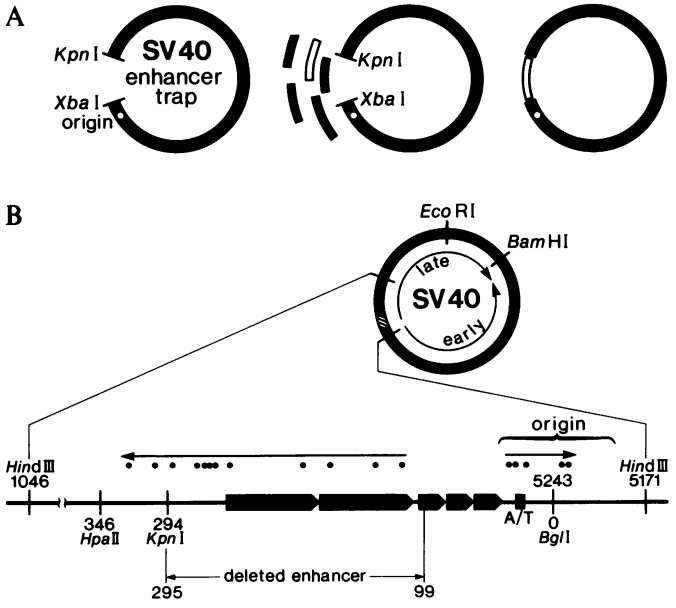

Fig. 1. The SV40 enhancer trap. (**A**) Schematic diagram of an SV40 enhancer trap experiment. Enhancer trap molecules are mixed with sonicated DNA to be investigated and are transfected into CV-1 monkey cells. *In vivo* recombination occurs and fragments of DNA with the ability to substitute for the SV40 enhancer give rise to viable SV40 recombinant virus. (**B**) Map position of the deletion in the enhancer trap molecule relative to the transcription/replication control region of wild-type SV40. The 21-bp and 72-bp tandem repeats are symbolized by bold arrows. Numbering of nucleotides is according to Tooze (1981). The positions of major early (Everett *et al.*, 1983) and late (Contreras *et al.*, 1982) RNA initiation sites are indicated by dots.

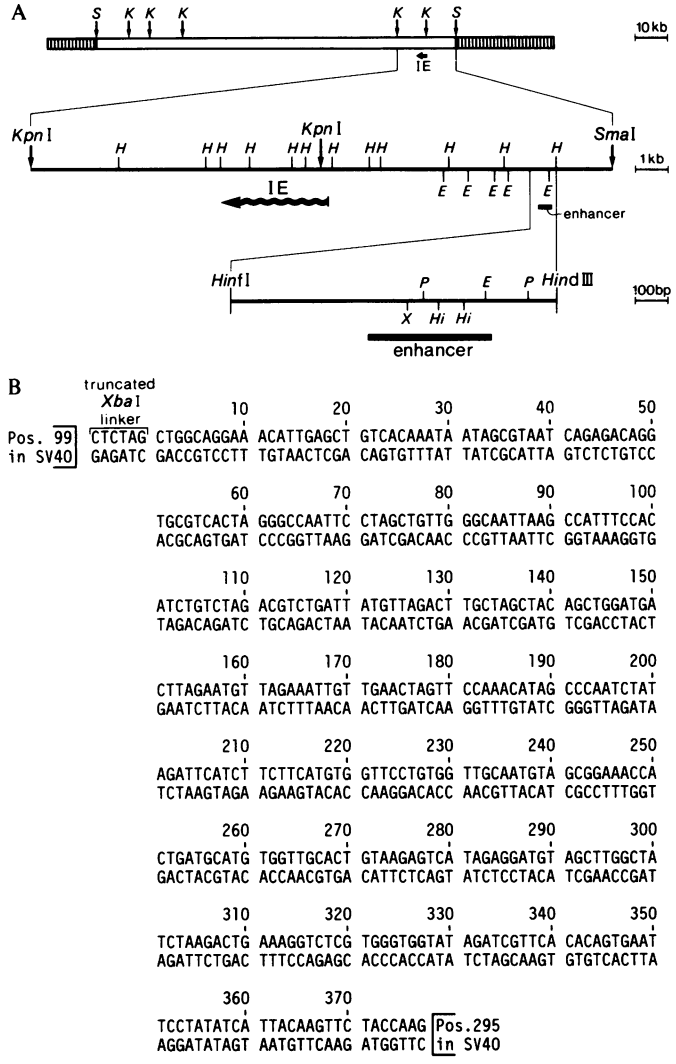

Fig. 2. The SV40-*H. saimiri* recombinant SVHS-2 (**A**) Map position of the functional *H. saimiri* L-DNA segment with enhancer activity, located within the righthand *Kpn*I/*Sma*I fragment (*Kpn*-E fragment). The enhancer sequence contained within recombinant SVHS-2 is indicated by a black bar. Restriction endonuclease cleavage sites: E = *Eco*RI, H = *Hind*III, Hi = *Hinf*I, K = *Kpn*I, P = *Pvu*II, S = *Sma*I, X = *Xba*I. The location of the single *H. saimiri* immediate-early (IE) gene and the direction of transcription are symbolized by an arrow. (**B**) Nucleotide sequence of the *H. saimiri* DNA contained within recombinant SVHS-2.

DNA (indicated by a black bar in Figure 2A). Sequencing of the SVHS-2 enhancer region showed that a stretch of 377 bp of *H. saimiri* DNA was inserted between base positions 99 and 295 of SV40, flanked on one side by a residual 6 bp of the *Xba*I-linker present in the SV40 enhancer trap (Figure 2B). Sequencing was also done in parallel with cloned *H. saimiri* virion DNA. The nucleotide sequences were found to be identical, demonstrating that the *H. saimiri* DNA substituting for the SV40 enhancer in recombinant SVHS-2 is a continuous stretch of genuine *H. saimiri* DNA.

Deletions in the enhancer-substituting sequence

Further experiments were performed to define more closely *H. saimiri* sequences required for enhancing activity. A recombinant virus, designated SVHS-8, was obtained by an enhancer trap experiment with the *Eco*RI/*Xba*I-subfragment of *H. saimiri* DNA contained within recombinant SVHS-2. The length and location of the *H. saimiri* segment substituting for the SV40 enhancer in SVHS-8 is shown in Figure 3. Of particular interest were recombinants that resulted from a co-transfection of the sonicated righthand *Kpn*I/*Sma*I-fragment (Kpn-E fragment) with the linear enhancer trap molecule. Another five clones of viable virus were isolated from this enhancer trap experiment. Two of these clones, designated SVHS-14 and SVHS-7, were mapped with restriction endonuclease and the inserts were sequenced. In the many previous enhancer trap experiments the SV40 recombinants had incorporated heterologous enhancers without any internal sequence rearrangements (Weber *et al.*, 1984; Boshart *et al.*, 1985; Weber and Schaffner, 1985; Dorsch-Häsler *et al.*, 1985; E.Serfling, A.Lübbe, K.Dorsch-Häsler and W.Schaffner, in preparation). In contrast, however, both clones SVHS-14 and SVHS-7 have a similar internal deletion as compared with the genomic *H. saimiri* DNA (Figure 3). One of the end points of the internal deletion in the *H. saimiri* segment is the same for both recombinants. Interestingly, the junctions between the *H. saimiri* DNA and the SV40 genome are identical for both clones, suggesting that both isolates were derived from a common ancestor and that the deletions were advantageous for the outgrowth of the two recombinant viruses. Figure 3 shows the relative map positions of the *H. saimiri* DNA inserts in the four sequenced recombinants.

S1 mapping experiments analogous to those described earlier (Weber *et al.*, 1984; Boshart *et al.*, 1985) proved that the *H. saimiri* segment fulfills all enhancer criteria. The genomic *H. saimiri Hind*III fragment of 1.6 kb containing the enhancer (Figure 2A) and the *Hind*III-C fragment of recombinant SVHS-14 were cloned into a downstream position to the genomic rabbit β-globin transcription unit in plasmid pβG (de Villiers *et al.*, 1982) and

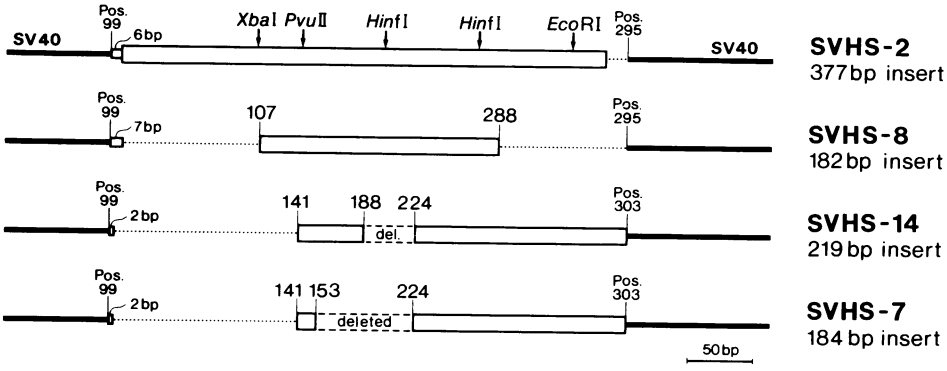

Fig 3. Map positions of *H. saimiri* enhancer DNA contained within four autonomously replicating SV40 recombinants. The nucleotide positions of *H. saimiri* inserts in recombinants SVHS-8, SVHS-14 and SVHS-7 are relative to the *H. saimiri* DNA contained in SVHS-2 (top). The residual base pairs from the *Xba*I-linker of the enhancer trap molecule are indicated for each recombinant at the left hand SV40/*H. saimiri* junction.

Table I. T-antigen gene expression measured by indirect immunofluorescence

Transfected DNA	Cell type				
	Experiment I		Experiment II		
	CV-1 (1 μg)	OMK (1 μg)	CV-1 (1 μg)	OMK (1 μg)	CV-1 (10 ng)
pSV3X	16.5% (3118/18900)	3.1% (456/14500)	18.4% (3912/21230)	3.7% (1470/39270)	1.4% (440/32505)
pET	None (−/14630)	None (−/16390)	None (−/9746)	None (−/42680)	None (−/39490)
SVHS-2	2.6% (605/23100)	0.01% (2/18810)	5.4% (695/12870)	0.009% (3/33550)	0.4% (167/38665)
SVHS-8	2.6% (495/18700)	0.08% (17/20570)	5.6% (610/10852)	0.08% (36/44550)	0.2% (80/37477)
SVHS-14	9.7% (1963/20130)	2.0% (276/13300)	15.4% (2145/13860)	2.0% (1035/51370)	1.1% (360/34155)
SVHS-7	11.8% (1683/14190)	0.7% (127/17930)	7.6% (1302/16940)	0.5% (246/48400)	0.5% (155/28769)

All transfections were done with 1 μg or 10 ng DNA of the various clones, as indicated below the cell type. The percentage of fluorescent cells and, in brackets, the extrapolated number of T-antigen positive cells and the extrapolated total number of cells is given. The total number of cells in a 8 x 8 mm area was extrapolated from counting 2−3 areas of 0.58 mm² each (200-fold magnification), that is, a total of 250−800 cells were counted. The number of fluorescent cells was counted either in the entire 8 x 8 mm area or in a defined section thereof, if the percentage of stained cells was high.

used for transient expression assays in HeLa cells. Both inserts strongly stimulated β-globin transcription in an orientation-independent fashion and over large distances. Compared with the SV40 enhancer present in a similar construct (recombinant pβGSV; Weber *et al.*, 1984), the *H. saimiri* enhancer is approximately half as strong (data not shown).

Activation of T-antigen expression

To estimate roughly the strength of their respective enhancer sequences, DNA of the recombinant clones SVHS-2, SVHS-8, SVHS-14, and SVHS-7 was transfected into CV-1 and owl monkey kidney cells by the DEAE-dextran procedure. CV-1 cells, which are permissive for SV40 growth, are kidney cells of the Old World monkey *Cercopithecus*, whereas kidney cells of the owl monkey (*Aotus trivirgatus*), a species of New World primates, are mostly used for culturing *H. saimiri*. T-antigen expression was monitored by indirect immunofluorescence after 2 days. As controls, plasmids containing either an enhancerless SV40 (pET-1) or a recombinant plasmid with three copies of the entire SV40 wild-type genome (SV3x) were transfected in parallel. For all clones investigated, transfection was more efficient with CV-1 than owl monkey kidney cells. Nevertheless, the results obtained with the various clones were in accordance for both cell lines (Table I). Obviously, the enhancing activity of clones SVHS-2 and SVHS-8 which contain genomic stretches of *H. saimiri* DNA is weaker than that of clones SVHS-14 and SVHS-7 which arose by *in vivo* deletion of short DNA segments within the enhancer region.

Search for sequence motifs in the H. saimiri enhancer sequence

We analysed the 377-bp enhancer of SVHS-2 for possible enhancer 'core' sequences (Weiher *et al.*, 1983). Six short related DNA stretches were found (Figure 4; only one sequence related to this motif was found in a stretch of similar length in the coding region of the *H. saimiri* immediate early gene; H.H.Niller, W.Bodemer and B.Fleckenstein, unpublished). One of the 'core' sequences of SVHS-2 was found to be deleted in recombinants SVHS-14 and SVHS-7 (Figure 5), which have a stronger enhancer than SVHS-2 and SVHS-8, as is evident in CV-1, and even more strikingly in owl monkey kidney cells (Table I and Discussion). Only weak homologies exist with three motifs typical for the enhancer region of human cytomegalovirus (Boshart *et al.*, 1985) and two important motifs of the adenovirus 5 E1A gene enhancer (Hearing and Shenk, 1983). However, sequences very closely related to the 'TAATGARATTC' consensus sequence of the immediate early upstream regions of herpes simplex virus type 1

(HSV-1; Mackem and Roizman, 1982; Murchie and McGeoch, 1982; Whitton *et al.*, 1983; Preston *et al.*, 1984), and also to a motif identified in the immunoglobulin heavy chain enhancer (Ephrussi *et al.*, 1985) were found (Figure 4). Three of the former overlap with the enhancer 'core' sequence (Weiher *et al.*, 1983). Finally, a computer search (Squires *et al.*, 1983) was done to identify tandemly repeated sequences and inverted repeats within *H. saimiri* DNA of SVHS-2. Only two tandem repeat motifs of 26 and 22 bp with limited homology (~75%; indicated by 1 and 2 in Figure 5), in addition to some short repeated motifs (not shown), have been found. Twenty one examples of inverted repetitions are also present in the enhancer segment, if all structures with a stem of at least 10 bp matching by at least 20 hydrogen bonds are considered (not shown). However, inverted repeat structures are not abundant in other viral enhancers, and a correlation between enhancing activity and stem-loop structures, if any, remains to be shown.

(1) HSV-1 immediate early upstream sequence

consensus:	TAATGA$^{A}_{G}$ATTC	(Preston et al., 1984)
+strand pos. 37	TAATCAGAGAC	
-strand pos.353	TAATGATATAG	
pos.111	TAATCAGACGT	

(2) Enhancer "core"

consensus:	TGTGG$^{AAA}_{TTT}$G	(Weiher et al., 1983)
+strand pos.217	TGTGGTTCC	
pos.226	TGTGGTTGC	
pos.259	TGTGGTTGC	
pos.323	GGTGGTATAG	
-strand pos.101	TGTGGAAATG	
pos.251	AGTGGTTTC	

(3) Immunoglobulin enhancer motif

consensus:	CAGGTGGC	(Ephrussi et al., 1985)
+strand pos.140	CAGCTGGA	
pos.215	CATGTGGT	
pos.257	CATGTGGT	
-strand pos. 98	GATGTGGA	

Fig. 4. Partial homologies between the *H. saimiri* enhancer and sequence motifs from other enhancer elements.

Discussion

Enhancer trap experiments and the biology of H. saimiri

We have used the previously successful enhancer trap approach (Weber *et al.*, 1984; Boshart *et al.*, 1985; Weber and Schaffner, 1985) to search for regulatory enhancer-type elements in the complete genome of *H. saimiri* by a shotgun strategy. This study shows that the enhancer trap system can be applied to efficiently select for functional enhancers from a large excess of non-enhancing DNA fragments. The virion DNA was sonicated and co-transfected together with the enhancerless SV40 genome into CV-1 cells without prior ligation. Recovery of SV40-like virus is due to very active intracellular ligation/repair mechanisms after uptake of linear DNA fragments.

The enhancer element found in the righthand L-region of *H. saimiri* is located 7 kb upstream of the DNA sequence coding for the single immediate early RNA of 2.5 kb (Bodemer *et al.*, 1984). As there are no indications that this transcript is spliced (W.Bodemer and B.Fleckenstein, unpublished), the enhancer may activate immediately early transcription over a long distance. Effects over several kilobases in the natural context have been observed for viral and cellular enhancer elements (Banerji *et al.*, 1983; Gillies *et al.*, 1983). However, it could also be that the *H. saimiri* enhancer serves another function, i.e., control of viral DNA replication, as has been demonstrated for the polyomavirus enhancer (de Villiers *et al.*, 1984). The fact that the enhancer-containing sequence is preserved in all lymphoid tumor cell lines transformed by *H. saimiri* (Desrosiers, 1981; Schirm *et al.*, 1984) is compatible with the hypothesis that this DNA segment has a role in the establishment and/or maintenance of the transformed phenotype.

Sequence analysis

An inspection of the *H. saimiri* enhancer sequence has revealed short homologies to several enhancer consensus motifs. Three stretches with good homology to the HSV-1 immediate early upstream region (consensus sequence TAATGARATTC) have been found (Figure 4). In HSV-1, this sequence seems to be an important motif of an enhancer-like element which is acted upon

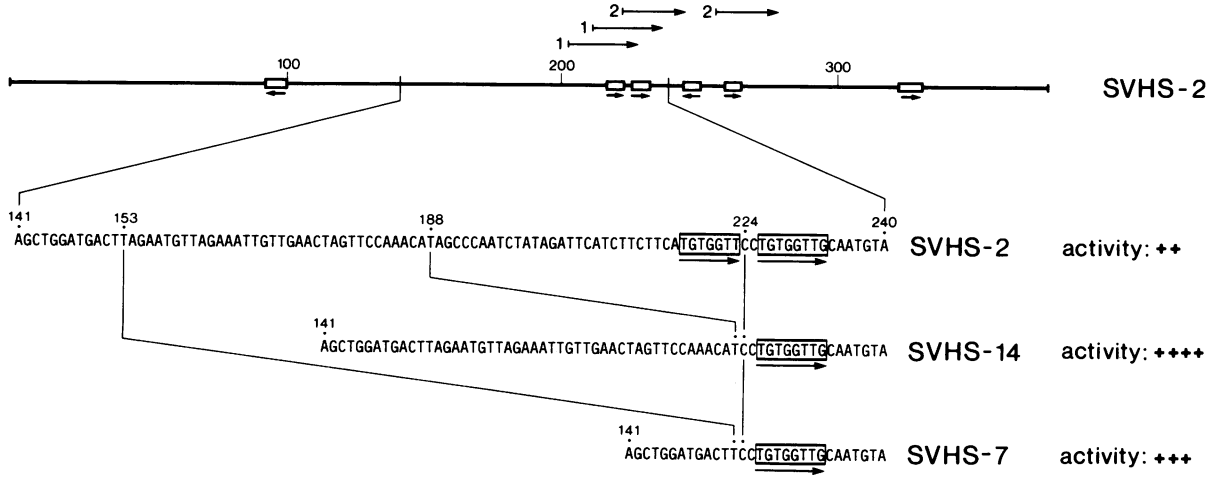

Fig. 5. Enhancer deletion variants. The enhancer 'core' sequences (Weiher *et al.*, 1983), are indicated by boxes, their orientation is given by the underlining arrows. Regions of tandem homologies are marked by arrows on top of the figure (1 and 2). The deletion in both recombinants SVHS-14 and SVHS-7 removes one of two closely spaced 'core' sequences. The enhancer activity in owl monkey kidney cells is indicated by plus signs. (For details see Table I.)

by a virion component (Preston *et al.*, 1984). However, no functional importance of these DNA stretches could be shown in preliminary superinfection experiments with *H. saimiri* in combination with transfection of DNA from recombinants SVHS-2, SVHS-8, SVHS-14 or SVHS-7 (S.Schirm, unpublished). We have also identified several copies of the enhancer 'core' sequence (Weiher *et al.*, 1983) and a putative enhancer consensus motif found in the immunoglobulin heavy chain enhancer (Ephrussi *et al.*, 1985). The former are particularly interesting, because one of two very closely spaced 'core' sequences is deleted in recombinants SVHV-14 and SVHS-7 and this deletion apparently augments enhancer activity. Sequence rearrangements within enhancers must be rare; in 12 independently obtained and sequenced recombinants from various enhancer trap experiments no deviation from the expected sequence has been observed (Weber *et al.*, 1984; Boshart *et al.*, 1985; Weber and Schaffner, 1985; Dorsch-Häsler *et al.*, 1985; E.Serfling, A.Lübbe, K.Dorsch-Häsler and W.Schaffner, in preparation). The observation that SVHS-14 and SVHS-7 both have deleted similar DNA segments and both have stronger enhancer activity than the two recombinants not containing any deletion suggests that SVHS-14 and SVHS-7 were selected due to a growth advantage. The deletion variants are most likely derived from a common ancestral recombinant because the *H. saimiri*/SV40 junctions at both the lefthand and righthand side are identical (Figure 3).

We speculate that there may be steric hindrance of protein factors binding to the two very closely linked enhancer 'core' sequences in SVHS-2 (Figure 5), since the sequence deleted in SVHS-14 and SVHS-7 apparently exerts a negative effect in the isolated enhancer incorporated in SV40. That hindrance may be relieved by a viral factor within a cell infected with, or transformed by, *H. saimiri*. So far, however, simple transfection of *H. saimiri*-infected cells with the SV40-*H. saimiri* recombinants has not improved the activity of the non-rearranged enhancer relative to the deleted enhancer (S.Schirm, unpublished). Nevertheless, the experiments with the *H. saimiri* enhancer suggest an approach to investigate other enhancers whose activity is subject to regulation. Based mainly on findings with the inducible enhancers of the metallothionein genes (E.Serfling, A. Lübbe, K.Dorsch-Häsler and W.Schaffner, in preparation), we conclude that regulated enhancers generally have a modular structure, being composed of several intermingled DNA motifs which either bind constitutive or regulatory protein factors. Binding of regulatory factors may be a prerequisite for a specific spatial arrangement of components which, as a complex, allow transcription stimulation by an enhancer. This may be achieved, for example, by overcoming steric hindrance (see above), or by bridging gaps between constitutive factors. In the non-induced state the regulatory sequences may exert a negative effect. Following this hypothesis (Serfling *et al.*, 1985; for review, see also Gluzman, 1985), it should be possible by genetic means to identify the DNA segment(s) responsible for regulated expression. We would expect that an enhancer segment deleted in a selection process performed in the absence of the regulatory stimulus normally is involved in binding a regulatory factor.

Materials and methods

Virus, cell culture

H. saimiri strain 11 (Falk *et al.*, 1972) was grown on monolayers of owl monkey kidney cells (Daniel *et al.*, 1976; Todaro *et al.*, 1978). For virion DNA preparation, virus particles were purified from the culture fluid (Fleckenstein *et al.*, 1975) and dissociated by N-lauroyl sarcosinate. The DNA was isolated by isopycnic centrifugation in CsCl-gradients (Fleckenstein and Wolf, 1974). Culturing CV-1 (*Cercopithecus* kidney) and HeLa cells followed standard conditions (Weber *et al.*, 1984).

DNAs

The SV40 'enhancer trap' (clone pET-1; Weber *et al.*, 1984) was released from the plasmid vector by cleavage with *Kpn*I and *Xba*I. In clone pβGSV (Weber *et al.*, 1984) the SV40 enhancer is inserted in plasmid pβG (de Villiers *et al.*, 1982) downstream of the genomic rabbit β-globin gene. The isolation of the genomic clones of *H. saimiri* have been described (Knust *et al.*, 1983). Plasmid cloning experiments were done by standard protocols (Knust *et al.*, 1983). Purification of cloned DNA followed the method of Ish-Horowicz and Burke (1981). SV40 virion DNA was isolated from infected cells by Hirt-extraction (1967).

Nucleotide sequencing followed the Maxam-Gilbert protocol (1980). The S1 nuclease analysis was performed with a β-globin gene clone from which the first intron had been deleted (a gift from D. Hans Weber, Zürich; described in Rusconi and Schaffner, 1981).

Transfection assays

Transfection by the calcium phosphate precipitation method (Graham and van der Eb, 1973; Wigler *et al.*, 1978) was modified according to Weber *et al.* (1984). The procedure for transfection with DEAE-dextran was according to Luthman and Magnusson (1983) with slight modifications (de Villiers and Schaffner, 1983). The protocol for the indirect immunofluorescence assay has been described in Banerji *et al.* (1981, 1983).

Quantitation of RNA by S1 analysis

HeLa cells were transfected by the calcium phosphate precipitation method. For RNA extraction cells were trypsinized and lysed in a buffer containing 0.5% Nonidet P-40, followed by digestion with Proteinase K and DNase I (Picard and Schaffner, 1983). The S1 nuclease mapping was done as described by Berk and Sharp (1977) and modified by Weaver and Weissmann (1979). A single-stranded, 5'-labeled DNA probe was used to map the initiation sites of the transcripts (Rusconi and Schaffner, 1981; de Villiers and Schaffner, 1981).

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