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Small fragments of herpesvirus DNA with transforming activity contain insertion sequence-like structures

(herpes simplex virus type 2/cytomegalovirus/morphological transformation/DNA sequence)

DENISE A. GALLOWAY, JAY A. NELSON, AND JAMES K. MCDOUGALL

Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104

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ABSTRACT **A 737-base-pair fragment of herpes simplex virus type 2 DNA with morphological-transforming ability was identified by transfecting into rodent cells deleted fragments of the left-hand end of the *Bgl* II N fragment region (map position 0.58–0.625), which were constructed *in vitro*. The transforming sequences lie within the coding region for a M_r 61,000 protein, but the fragment itself does not appear to specify a viral polypeptide. Contained within the transforming fragment are sequences that can be drawn as a stem–loop structure flanked by direct repeats, similar to an insertion sequence-like element. An insertion sequence-like structure was also found in a small fragment of human cytomegalovirus DNA that has transforming activity. Possible mechanisms of herpesvirus transformation are discussed.**

All of the human herpesviruses have demonstrated oncogenic potential. Both herpes simplex viruses (HSV-1 and HSV-2) (1, 2), cytomegalovirus (CMV) (3), and varicella zoster virus (VZV) (4) can alter the growth properties of rodent cells in culture, usually to a tumorigenic phenotype. Attempts to identify the viral genes that are responsible for HSV-mediated morphological transformation gave surprising results. First, although HSV-specific antigens have been reported in a number of transformed cells (5–8), no viral protein was invariably expressed. Second, when subgenomic fragments of DNA were used to alter cells, two different regions of the HSV-2 genome showed transforming ability (9–11), neither of which was homologous to the HSV-1 transforming region (9, 12). And third, there have been reports that the quantity and complexity of viral sequences retained by the transformants change with passage of the cell lines (13, 14) and that the transformed phenotype can even persist in the absence of any viral sequences (15–19).

Exposure to the HSV-2 *Bgl* II N fragment (map positions 0.58–0.625) has led to the stable establishment of transformed rodent cells (9, 10) by using as a selective assay focus formation in low serum or colony formation in methylcellulose. The transformants show altered growth properties in culture, form tumors in nude mice and in their syngeneic hosts, and frequently (in approximately 75% of the cell lines) retain a subset of the *Bgl* II N sequences in less than one copy per cell. The polypeptides that are encoded by the *Bgl* II N fragment have been identified by hybrid-arrested translation (20) and hybrid-selection translation (21). The latter approach mapped the location of four polypeptides to this region. The sequences encoding three of the proteins of M_rs 38,000, 56,000, and 61,000 fall entirely within the *Bgl* II N fragment. The message specifying a protein of M_r 140,000 spans the left-hand *Bgl* II site; in fact, DNA sequence analysis of this region (22) has shown that most, though not all, of the coding sequences for that protein are within the adjacent *Bgl* II C fragment. Recently, it has been suggested that either the M_r 140,000 protein or the M_r 38,000 protein, or both, specify the virally encoded ribonucleotide reductase (23).

The transfection of rodent cells with overlapping cloned segments of the CMV genome strain AD169 identified a transforming fragment of 2.9 kilobases located near the right-hand end of the genome (24). In experiments similar to those reported here, a series of deleted fragments were constructed from the CMV fragment, and, when used to transform rodent cells, a 490-base-pair (bp) fragment was identified (25). DNA sequence analysis revealed that there are multiple stop codons in all reading frames, so that it is unlikely that the CMV transforming fragment encodes a protein.

MATERIALS AND METHODS

Cells and Culture Methods. Cultures of primary rat cells were obtained by trypsinizing 14-day Wistar white rat embryos. NIH 3T3 cells were obtained from R. Kucherlapati (University of Illinois Medical Center). Cells utilized for transfection were passed no more than twice and grown in the Dulbecco modification of Eagle's medium (DME medium; GIBCO) with 10% fetal calf serum (Hyclone) on plastic tissue culture plates (Falcon).

DNA Transfection Assays. Transfection assays involved precipitation of 10 μg of recombinant plasmid DNA with 10 μg of calf thymus carrier DNA (Sigma) by the calcium phosphate technique (26) onto subconfluent monolayers of cells as described (10). After 24 hr the cells were passaged 1:3 and put into selective media. For selection in methylcellulose, cells were trypsinized and seeded at 1×10^5 cells per 100-mm plate containing DME medium with 10% fetal calf serum and 1.2% methylcellulose (Fisher). For selection in low serum, cells were trypsinized, seeded at 1×10^5 cells per 100-mm plate and allowed to reach confluence in DME medium with 10% fetal calf serum. After reaching confluence, medium with 2% fetal calf serum was added and changed twice weekly.

Construction of Recombinant Molecules. Construction of the recombinant plasmid pP2 derived from pDG401 has been described (21) as has the construction of the plasmid pmtrIIa and a series of deleted fragments (22).

The *Bam*HI/*Hin*dIII fragment of pmtrIIa also was cleaved with a variety of restriction enzymes and ligated into the appropriately cleaved replicative form of the vectors M13pm7, mp8, mp9, mp10, and mp11 as described (22).

DNA Sequence Analysis Methods. Sequence analysis by dideoxynucleoside triphosphate chain termination (27) was performed by utilizing as template the single-stranded M13 phage DNAs (28) as described (22).

The deletion fragments were sequenced by the method of Maxam and Gilbert (29). Approximately 10 μg of each deletion mutant was digested with *Bam*HI or *Hin*dIII and labeled at the 3′ end with 10 units of reverse transcriptase (Life Sci-

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Abbreviations: IS, insertion sequence; bp, base pair(s); HSV-1 and -2, herpes simplex virus types 1 and 2; CMV, cytomegalovirus.

ences, St. Petersburg, FL) in the presence of [^{32}P]dCTP, [^{32}P]dTTP, 0.5 mM dATP, and 0.5 mM dGTP as described (30).

RESULTS

Precise Localization of the HSV-2 Transforming Sequences. The *Bgl* II N fragment cloned into pBR322, which has been described previously as pDG401 (10), was cleaved with restriction endonucleases *Hin*dIII, *Bam*HI, *Bst*EII, *Sst* I, *Sal* I, and *Pst* I (Fig. 1), and the digested DNAs were precipitated onto monolayers of either NIH 3T3 cells or Wistar white rat embryo cells. Of these enzymes, only cleavage with *Sst* I inactivated transformation (data not shown). The four individual *Pst* I segments were recloned into pBR322 and used in transformation assays. The results shown in Table 1 indicated that only P2, containing the left-hand end of the *Bgl* II N fragment, showed significant transforming activity. Further experiments concentrated on precisely defining the transforming sequences within the left-hand *Pst* I fragment.

In the initial description of pDG401 (10), it was noted that a deletion between the *Bam*HI site and the *Bgl* II site had occurred (see Fig. 1), but the extent of the deletion was unknown. To determine this, two fragments—one from pDG401 and one from the *Bam*HI E fragment containing the authentic *Bam*HI–*Bgl* II sequences—were cloned into M13 phage vectors. DNA sequence analysis revealed that all of the viral sequences leftward of the *Bam*HI site had been deleted from pDG401 and that the appropriate sequences of pBR322 were adjacent to the *Bam*HI site (data not shown). Therefore, the 550 bases at the left end of the *Bgl* II N fragment were not necessary for transformation.

To obtain shorter segments of the left-hand *Pst* I fragment, deletions were constructed *in vitro*. They were sequenced directly to determine the extent of the deletion and tested for transforming activity. The collection of 10 deleted fragments used in this study is shown in Fig. 2, and results with the fragments are shown in Table 1. In summary, all of the deletions from the *Bam*HI end of the fragment remained active in transformation, the smallest fragment, BC24, being 794 bp. The only deletion starting at the *Hin*dIII end that retained transforming activity was HA13, from which 57 bases had been deleted. This placed the transforming gene within a 737-bp fragment just leftward of the *Pst* I site at approximately position 0.60 on the HSV-2 genome. The deletion fragment BC24 has been tested extensively; in this case as well as for all of the other deletions where multiple experiments are available, any fragment that scored as a positive was capable of both focus and colony formation and could transform either NIH 3T3 or rat embryo cells.

The efficiency of transformation (1–10 colonies or foci per μg of DNA per 10^6 cells) was surprisingly low for a cloned gene. It is also interesting that the efficiency did not improve by using smaller fragments from the original level observed with the intact *Bgl* II N fragment (10). In Table 1, experiment 7, transformants were obtained when DNA from two primary transformants, 401 NIH-0-1 or P2N2, was used to transform NIH 3T3 cells. Secondary transformants were obtained with an efficiency compatible with the possibility that the initially transformed cells had indeed acquired or activated a transforming gene—i.e., 10^6 more efficient than the primary event.

Fine Structure Analysis of the Transforming Region. The exact nucleotide sequence from the *Bam*HI site (position 0.585) to the *Pst* I site (position 0.60) was obtained by sequencing both the deletions and the fragments cloned into M13. DNA sequence analysis of the region revealed the following features: (*i*) the complete fragment from the *Bam*HI site to the *Pst* I site is 2050 base pairs long; (*ii*) the coding region for the protein (M_r = 38,000) and the polyadenylylation signal for the 1.2-kbp message are located within the *Bam*HI/*Pst* I fragment but are not contained with the transforming fragment BC24. The details of this sequence have been presented elsewhere (22), and a summary of the organization of the *Bgl* II N fragment is shown in Fig. 1; and (*iii*) the start of the deletion BC24, which was active in transformation, occurs 26 nucleotides to the right of the first nucleotide of the polyadenylylation signal for the RNA encoding another viral protein (M_r = 61,000). The DNA sequence of this fragment is shown in Fig. 3.

Several interesting features have emerged from the DNA sequence analysis of BC24. The G+C content reflects that of the overall genome in that it is 68% G+C rich. The 3′ region of the message that encodes the M_r 61,000 polypeptide is located in BC24, although the precise end of the message is outside BC24. The putative termination codon for the protein is located 38–40 nucleotides from the border of the fragment, followed by an open reading frame throughout the rest of the fragment, which codes for 251 amino acids, somewhat less than one-third of the predicted size of the protein. All of the other reading frames have at least 1 and as many as 10 termination codons. No obvious transcriptional regulatory

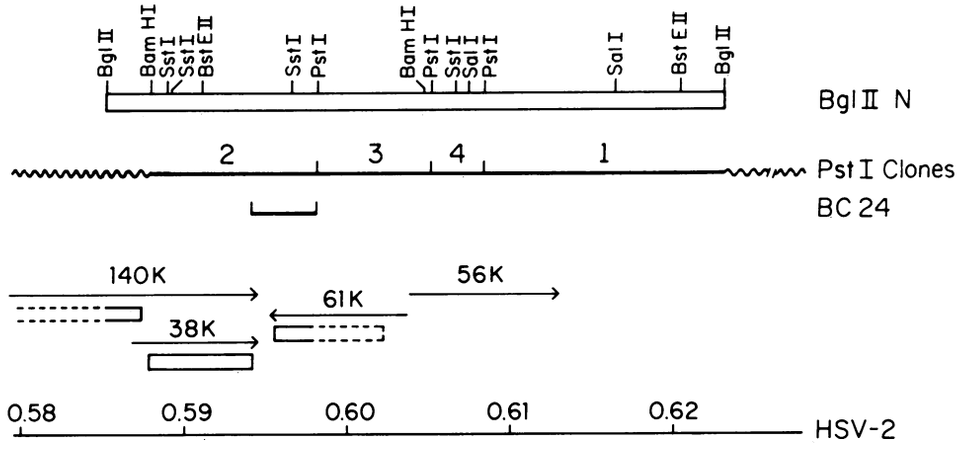

FIG. 1. A summary of features contained within the HSV-2 *Bgl* II N fragment. The top box indicates the *Bgl* II N fragment and the sites of cleavage by five restriction enzymes. The second line indicates the four *Pst* I subclones derived from pDG401 (the wavy line indicates the pBR322 vector sequences), below which is shown the location of the transforming fragment BC24. Arrows indicate transcripts that have been mapped to the region and the size of the proteins translated *in vitro* by them. The closed boxes below the arrows indicate open reading frames that have been identified by DNA sequence analysis. The bottom line shows the map coordinates along the HSV-2 genome.

Table 1. Transformation of rodent cells with fragments of HSV-2 *Bgl* II N fragment DNA

Exp.	Cell* type	Selec.† method	Number of transformants‡																		
			pBR322	pDG401	P1	P2	P3	P4	pmtrIIa	BC24	BC10	BC3	BA23	BA4	HA13	HA16	HB8	HB9	HB15	401 NIH-01	P2N2
1	N	C	2	9	2	11	0	0	—	—	—	—	—	—	—	—	—	—	—	—	—
2	R	C	3	13	3	38	3	4	—	—	—	—	—	—	—	—	—	—	—	—	—
3	R	F	0	—	—	—	—	—	23	12	—	—	—	—	—	—	—	—	0	—	—
4	N	F	6	10	—	—	—	—	—	—	14	7	21	14	0	0	0	0	—	—	—
5	R	F	2	12	—	—	—	—	—	—	9	7	—	—	3	—	0	0	—	—	—
6	N	F	1	10	—	—	—	—	—	12	—	—	—	—	—	—	—	—	1	—	—
7	N	C	0	8	—	—	—	—	—	8	—	10	—	—	7	—	—	—	1	6	7

*Cell type: N, NIH 3T3; R, Wistar white rat embryo.
†Selection method: C, colony formulation in methylcellulose; F, focus formation in low serum.
‡Number of F or C per two 60-mm dishes of transfected cells.

elements such as a canonical "TATA" sequence or "CAAT" sequence (31) were observed.

A striking feature of the DNA sequence is that a stem–loop structure bounded by direct repeats can be formed from sequences contained within the small transforming fragment BC24. The heptanucleotide T-C-C-G-G-G-G, repeated at positions 324–330 and 477–484, flanks an 8-bp stem with one mismatch and a 129-base loop. Contained within the loop is the recognition site for cleavage by *Sst* I (position 410–415), an enzyme that rendered the DNA inactive in transformation assays. This insertion sequence (IS)-like structure is shown in Fig. 4.

Are IS-Like Sequences Involved in Transformation by Other Herpesviruses? Similar experiments to those reported here have identified a 490-bp fragment of human cytomegalovirus (hCMV) DNA strain AD169 that is able to transform rodent cells to a tumorigenic phenotype (25). In that case, the restriction endonuclease *Eco*RI inactivates transformation, and DNA sequence analysis has located the site 21 nucleotides from the right-hand border of the fragment. Examination of the DNA sequence around this region detected sequences that can be drawn as a stem–loop structure, as shown in Fig. 4. The octanucleotide C-T-T-G-T-G-T-T is repeated as C-T-T-G-T-A-T-T and flanks a 12-bp stem containing two mismatches and a 118-base loop. The *Eco*RI site is located at the top of the stem going into the loop. Despite the similarity of the HSV-2 and CMV IS-like structures, there is no DNA sequence homology between the two; in fact the CMV element is very A+T rich (70%), whereas the HSV-2 element is very G+C rich (76%). Although this difference in the base composition of the structures may reflect an underlying difference in the precise mechanism by which these two herpesviruses transform, the fact that fragments of viral DNA representing less than 0.2% of their respective viral genomes have been identified as active in transformation assays and the fact that both fragments contain sequences that are able to form stem–loop configurations provides circumstantial evidence to suggest involvement of these structures in transformation.

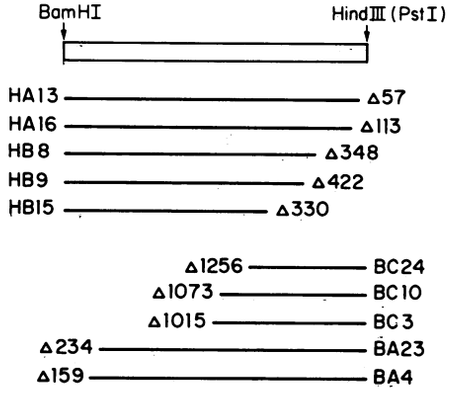

FIG. 2. A map of the deletion fragments of pmtrIIa. The top box denotes the viral insert of pmtrII from the *Bam*HI site to a *Hin*dIII site that was constructed *in vitro* at a *Pst* I site. Fragments in the HA and HB series were shortened from the *Hin*dIII site and those in the BA and BC series were shortened from the *Bam*HI site. The number of nucleotides deleted is shown for each fragment.

Several groups (9, 12) have shown that the HSV-1 transforming genes are located within map coordinates 0.32 and 0.41 on the viral genome and that this region has no homology to the HSV-2 transforming region. Equally, experiments to identify a transforming gene around position 0.60 on the HSV-1 genome have been consistently negative, despite the genetic similarity to the HSV-2 region. As part of a larger study on the genetic relatedness of HSV-1 and HSV-2 (unpublished data), DNA sequence from HSV-1 was obtained that encompassed the DNA encoding the 3′ end of the message for the HSV-1 M_r 61,000 protein. Interestingly, at one point the DNA sequence diverges dramatically and, as shown in Fig. 4, the sequence from HSV-1 cannot be drawn as an IS-like structure. The direct repeat in HSV-2, T-C-C-G-G-G-G, is found in one copy in HSV-1 as T-C-T-G-G-G-G, but the sequence in the position for the second repeat reads C-G-G-G-A-T-C. One portion of the stem is identical, C-G-T-C-G-T-G-G, but the other part of the stem reads G-T-A-A-C-G-T-C-C. Analysis of the HSV-1 DNA sequence did not reveal any other sequences in this region that are capable of forming a stem–loop structure. These results are suggestive but not proof of a correlation between the presence of IS-like sequences and the ability of a fragment of herpesvirus DNA to transform cells.

Characterization of Transformed Cells. In general the properties of the cells transformed by pmtrIIa or any of the deletions derived from that plasmid are identical to the properties we have described for cells transformed by the entire *Bgl* II N fragment (10). All of the cell lines derived from NIH 3T3 cells are able to grow to high saturation densities (5–10 times parental cell density) and grow well in low serum (2–3 times parental cell density). The parental rat embryo cells were unable to grow in 1% serum, but the transformants grew at an appreciable rate. Growth to high saturation densities was less marked in the rat embryo transformants but reached about 3 times the density of the parental cells. The efficiency of plating in methylcellulose was variable and ranged from 0.05% to 80%. The tumorigenicity of these cell lines has not yet been determined, but in the cells transformed by *Bgl* II N and pP2, there was a good correlation

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                                                                    60
CGGGAGGGGA TGGGGGCGCG CGTTTCCTCC GTTCCGGCTA CTCGTCCCAG AATTTAGCCA

                                                                   120
GGACGTCCTT GTAAAACGCG GGCGGGGGCG CGTGGGCCCA CAGCTGCGCC AGAAACCGGT

                                                                   180
CGGCGATGTC CGGGGCGGTG ATATGCCGAG TCACGATGGA GCGCGCTAAA TCTTCGTCGC

                                                                   240
GGAGGTCCTG ATAGATGGGC AGTCTTTTTA GAAGAGTCCA GGGTCCCCGC TCCTTGGGGC

                                                                   300
TGATAAGCGA TATGACGTAC TTGACGTATC TGTGCTCCAC CAGCTCGGCG ATGGTCATCG

                                                                   360
GATCGGGCAG CCAGTCCAGG GCCTCCGGGG CGTCGTGGAT GACGTGGCGG CGACGTCCGG

                                                                   420
CGACATAGCC GCGGTGTTCC GCGACCCGCT GCGCGTTGGG GACCTGCACG AGCTCGGGCG

                                                                   480
GGGTGAGTAT CTCCGAGGAG GACGACCGGG CGCCGTCGCG CGGCCCACCG GCGACGTCCG

                                                                   540
GGGGCTGGAG GGGGGGTCT TCTTCGTAGT CGTCCTCGCC CGCGATCTGT TGGGCCAGAA

                                                                   600
TTTCGGTCCA CGAGATGCGC GTCTCGAGGC CGACCGGGGC CGCGGTCAGC GTAGGCATGC

                                                                   660
TCTCCAGGGA GCGCGAGTTG GCGCGCTCCC CGCCGGGCCG CCCGGCGGGC CTGGGATCGG

                                                                   720
CTCGGGGCGG TCCAGTGACA CTCGCGCAGC ACGTCCTCGA CGGACGCGTA GGTGTTATTG

                                                                   780
GGGTGCAGGT CTGTGTGGCA GCGGACGAAC AGCGCCAGGA ACTGCGGGTA ACTCATCTTG

AAGTACCCTG CAG
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FIG. 3. The DNA sequence of the transforming fragment BC24. Sequence from the strand encoding the M_r 61,000 polypeptide is presented, with the left to right organization the same as the prototype viral arrangement. The potential termination codon is underlined (38–40) as are the sequences that form the direct repeats (324–300 and 477–484); the inverted repeats which form the stem (331–338 and 469–476) are underlined twice. The last six nucleotides are the authentic viral *Pst* I site.

between growth in semisolid media and tumorigenicity. The morphology of the cell lines was also variable, with some lines appearing to be fibroblastic while others looked to be more epithelial, and the number of refractile cells also varied.

Several lines of both HSV-2- and CMV-transformed cells were examined for the presence of viral sequences. There were clearly examples of cell lines that had retained at least a subset of the viral fragment used to transform the cells (unpublished results). Equally, there were cell lines in which no viral sequences could be detected. It is difficult to determine whether these transformants might be the result of a truly "hit and run" mechanism or whether only a small stretch of DNA was retained, less than can be detected by our hybridization techniques.

DISCUSSION

Experiments aimed at defining the exact sequences within the *Bgl* II N fragment of HSV-2 DNA that have transforming activity have identified a 737-bp segment that can convert rodent cells to an anchorage-independent phenotype. There is no evidence that this fragment encodes a polypeptide, indicating that there is at least one mechanism by which HSV-2, and perhaps herpesviruses in general, can initiate morphological transformation without the involvement of a viral protein. These results indicate that HSV-2 can transform cells by a mechanism not previously described for a DNA tumor virus and explains some of the unusual phenomena associated with herpesvirus transformation.

One possibility is that the herpesvirus fragments have enhancer activity. It was first demonstrated that a 72-bp segment of simian virus 40 DNA 100–175 nucleotides upstream of the start of the early mRNAs was essential for their expression (32, 33). Analogous elements have been found in other viruses and in human DNA, and there is evidence that the various elements may have different host-cell specificities (34). Experiments to define the critical nucleotides for enhancing activity have suggested a consensus sequence of G-T-G-G-A-A-A or G-T-G-G-T-T-T (35), with the G following the T an essential nucleotide. Within the HSV-2 transforming fragment, the sequence G-T-G-G-A-T (335-340) is homologous to the consensus sequence, and the sequence G-T-G-A-G-T-A-T (423-430) is similar. Within the CMV fragment the sequence G-T-G-A-T-T-T is present. However, these homologies may be coincidental.

Another type of sequence was recently described that activates the expression of heterologous genes. Fried *et al.* (36) described the isolation of a fragment of mouse cell DNA that could substitute for the 5′ expression sequences of the polyoma-transforming region. One of the striking features of the sequence is the repeated decamer C-T-T-C-C-G-G-G-A-C, which is very similar to the sequences that flank the stem–loop in the HSV-2 transforming fragment, C-C-T-C-C-G-G-G-G-C and C-G-T-C-C-G-G-G-G-C, and suggests the possibility that herpesvirus transformation may be mediated by DNA sequences that modulate the expression of cellular genes. In mouse cell DNA, the repeated decamer is separated by 15 nucleotides that cannot pair to form a stem, which might suggest that the direct repeats in the herpesvirus fragments, rather than the stem–loop structures, are important in transformation.

The possible involvement of an IS-like element in the activation of a cellular oncogene was first described in a mouse

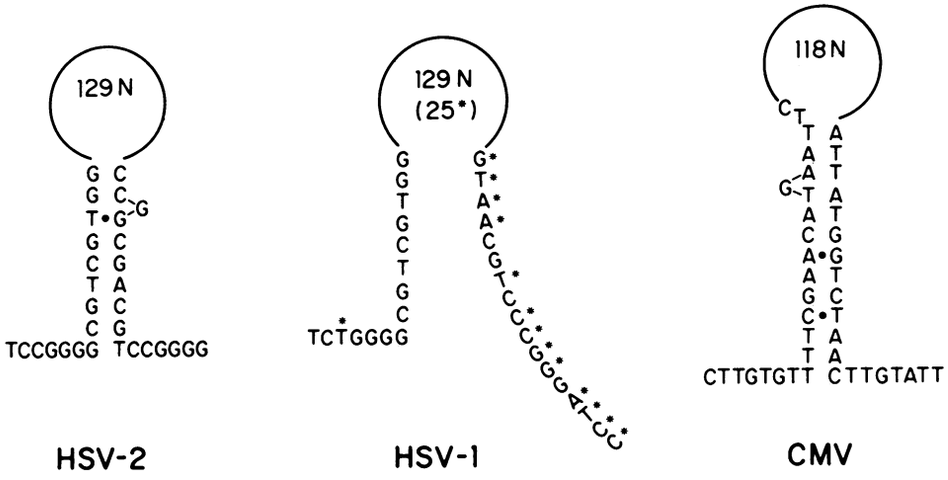

FIG. 4. The IS-like structures found in the HSV-2 and CMV transforming fragments. The potential IS-like structures are shown, and a dot indicates a mismatched pair in the stem. The homologous region of HSV-1 is shown, and the asterisks denote nucleotides that differ between HSV-1 and HSV-2.

myeloma (37) in which the 5′ end of the cellular c-*mos* gene had been replaced by a segment of DNA from the long terminal repeat of the intercisternal A particle gene (38). Contained within the inserted fragment were sequences that could be drawn as a stem–loop structure flanked by direct repeats. Whether the herpesvirus fragments are active in DNA transposition and, thus, can activate cellular oncogenes remains to be seen. The potential for an IS-like element to be involved in the initiation of transformation is not limited to activating the expression of cellular oncogenes. Another possibility is that the herpesvirus DNA fragments act in a more general fashion as a mutagen by integrating into, and perhaps excising imprecisely from, cellular genes at random. The rate of mutation at the hypoxanthine phosphoribosyltransferase *HPRT* locus has been measured in cells exposed to UV or neutral red-inactivated HSV-1 (39), and the partially inactivated virus was found to be as potent a mutagen as the chemical carcinogen 4-nitroquinoline-1-oxide. The mechanism by which HSV can act as a mutagen is unknown, but the possibility that IS-like sequences of viral DNA are involved should be considered.

Transformation mediated by small fragments of DNA represents a novel pathway for DNA tumor viruses but explains many of the unusual phenomena associated with herpesvirus transformation. First, the reason that no viral antigen(s) is consistently expressed in HSV-transformed cells becomes clear. When cells are transformed by using large segments of the genome, some sequences may fortuitously become associated with and expressed in the transformed cell genome. Second, the observation that viral sequences retained by transformed cells change in quantity and complexity during passage of the cell lines probably reflects the fact that there is no selective pressure to maintain a great deal of viral DNA. Third, the finding that the transforming genes of HSV-1 and HSV-2 are not located colinearly despite the genetic similarity of these regions of the genome can be explained by diversity at the level of nucleotide sequence, which is not reflected in the gene products encoded. And fourth, the low efficiency of transformation seen with these fragments would be expected because the random nature of activating a cellular oncogene or mutating a gene that affects a cell's morphological phenotype would be less frequent than the ease with which a fragment of viral DNA encoding a transforming protein and its regulatory sequences would be expressed. Consistent with this model is the observation that DNA from the cells initially transformed with small herpesviral fragments is efficient in second rounds of transformation.

The next important step in unraveling the oncogenic potential of HSV-2 and CMV will be to determine the precise mechanism by which these small fragments of viral DNA mediate transformation.

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