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The immediate-early mRNA that encodes the regulatory polypeptide V_{mw} 175 of herpes simplex virus type 1 is unspliced

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The structure and precise map location of the 4.1-kb herpes simplex virus type 1 (HSV-1) immediate-early mRNA (IE mRNA-3) that encodes a regulatory polypeptide, with an approximate mol. wt. of 175 000, has been determined. Nuclease S1 digestion procedures using labelled virus DNA probes have shown that IE mRNA-3 is unspliced and maps entirely within the TR_S/IR_S genome regions. DNA sequences at the 5′ and 3′ ends of IE mRNA-3 have been examined and regulatory signals involved in initiation of transcription and polyadenylation have been identified. No other mRNA is known to map within the region coding for IE mRNA-3.
Key words: HSV-1/IE mRNA-3/V_{mw} 175/TR_S/IR_S

Introduction

The process of virus transcription in cells infected with herpes simplex virus (HSV) has been divided into three phases namely immediate-early (IE), early, and late (Clements *et al.*, 1977; Jones and Roizman, 1979; Swanstrom and Wagner, 1974). The IE class comprises five major mRNAs, designated IE mRNAs-1 to -5 (Clements *et al.*, 1979). IE transcription does not require prior virus polypeptide synthesis. However, subsequent virus transcription (both early and late) is dependent upon the expression of at least one IE polypeptide, namely that encoded by IE mRNA-3. This polypeptide which has a mol. wt. of ~175 000, has been variously referred to as V_{mw} 175 (Marsden *et al.*, 1976) or ICP4 (Honess and Roizman, 1974). Temperature-sensitive mutants with lesions in the V_{mw} 175 gene express only IE mRNAs and IE polypeptides at the non-permissive temperature (Watson and Clements, 1978; Dixon and Schaffer, 1980). Further examination of one such mutant (*ts* K) has shown that shift up to the non-permissive temperature, even after early or late transcription has commenced, results in reversion to the IE transcriptional phase (Watson and Clements, 1980). Preston (1979), by using *ts* K, has shown that the IE polypeptide V_{mw} 175 is directly responsible for activation of the virus pyrimidine deoxyribonucleoside kinase gene at the transcriptional level. The mutation in *ts* K has been identified as a single base alteration located in the V_{mw} 175 coding sequences (Murchie *et al.*, in preparation). Thus, a functional V_{mw} 175 polypeptide is required to maintain virus transcription and also to maintain the post-IE transcriptional phases. The V_{mw} 175 gene is therefore of particular importance to studies on the control of virus gene expression.

The HSV genome consists of two unique DNA regions (U_L and U_S) which are flanked and joined by different sets of inverted repeats termed TR_L/IR_L and TR_S/IR_S, respectively (Sheldrick and Berthelot, 1974; Hayward *et al.*, 1975).

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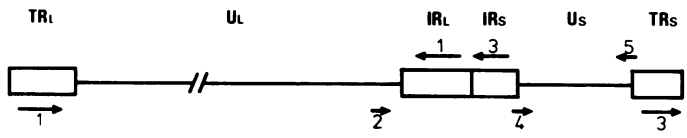

Fig. 1. Location and direction of transcription of the five major HSV IE mRNAs.

Previous mapping experiments have shown that the gene specifying IE mRNA-3 maps entirely within the TR_S/IR_S regions and thus is present as two copies in the HSV genome (Watson *et al.*, 1979; Anderson *et al.*, 1980; Mackem and Roizman, 1980). Figure 1 summarizes the map positions and the directions of transcription of the five major IE mRNAs.

The present study describes the structure of IE mRNA-3 and precisely locates the 5′ and 3′ ends of this mRNA. This information is correlated with DNA sequence data for these genome regions (Murchie and McGeoch, 1982; Davison and Wilkie, 1981).

Results

Structure of IE mRNA-3

Our initial experiments used HSV-1 genomic DNA, which had been ^{32}P-labelled *in vivo*. This was hybridized to IE cytoplasmic mRNA and the nuclease S1-digestion products were analysed on neutral and two-dimensional gels (Figure 2). Several nuclease S1-resistant bands were observed on the neutral gel, the largest of which had the size expected of a hybrid formed by the 4.1-kb IE mRNA-3. The nuclease S1-resistant material was also subjected to two-dimensional analysis. Bands formed by spliced mRNAs would migrate faster in the alkaline (denaturing) dimension than in the neutral dimension and therefore would appear below a diagonal formed by intact material (see Favaloro *et al.*, 1980). However, on the two-dimensional gel, the largest nuclease S1-resistant band migrated to a position on the diagonal indicating the absence of splices from within the central portion of IE mRNA-3. This form of analysis would not be expected to detect a splice located close to either end of the mRNA.

Location of the 3′ end

Southern blot hybridisation of ^{32}P-labelled cDNA made against HSV-1 IE mRNAs, using the procedure described by Clements *et al.* (1979), had located the 3′ end of IE mRNA-3 within *Bam*HI q and k. This procedure generates only short stretches of cDNA complementary to the 3′ ends of the IE mRNAs. To locate the 3′ end more accurately, ^{32}P-labelled cDNA was hybridised to *Sma*I-digested *Bam*HI k (Figure 3, track 3). The cDNA hybridised predominantly to *Sma*I a, indicating that the 3′ end was located within this fragment. The hybridisation of labelled total IE mRNA to *Sma*I c (Figure 3, track 2) was due to the 5′ end of IE mRNA-1 which extends into this fragment. The 3′ end of IE mRNA-1 lies in the neighbouring *Bam*HI b fragment.

Using 3′-labelled *Bam*HI k (Figure 7) as a probe in hybridisations with total IE cytoplasmic mRNA, a single

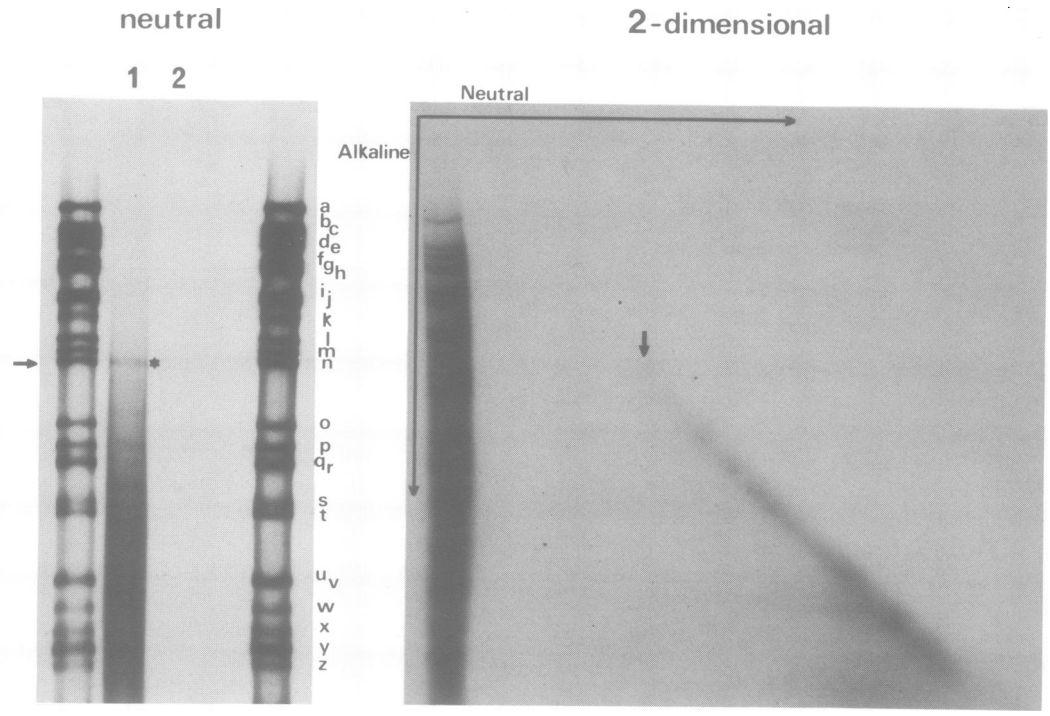

Fig. 2. Structural analysis of IE mRNA-3. *In vivo* ^{32}P-labelled HSV-1 DNA was hybridised to: (1) 20 μg of polyadenylated IE cytoplasmic RNA, (2) 20 μg of polyadenylated mock-infected cytoplasmic RNA. The nuclease S1-resistant material was electrophoresed on a 1.5% (w/v) neutral agarose gel. Sample (1) also was analysed on a 1.5% (w/v) two-dimensional agarose gel. *Bam*HI-digested HSV-1 DNA was used as a size standard.

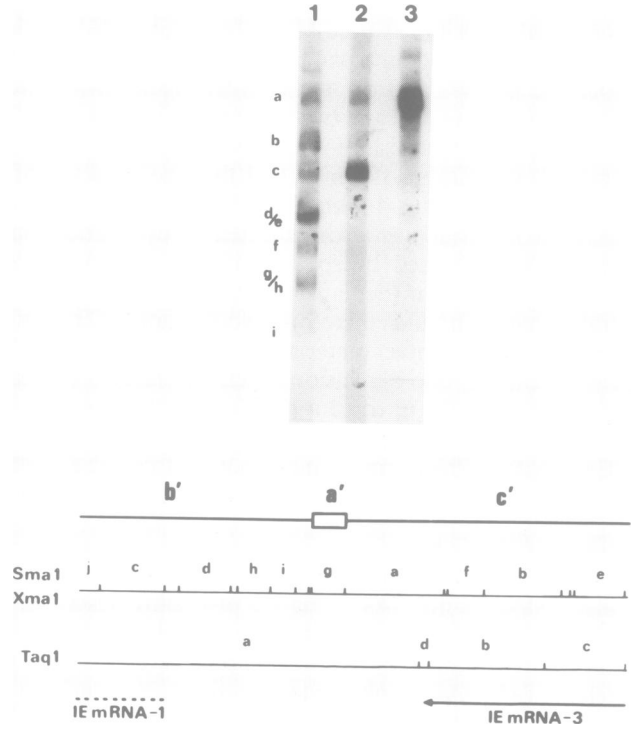

Fig. 3. Fluorographs of ^{32}P-labelled samples hybridised to fragments generated by *Sma*I digestion of *Bam*HI k DNA. Hybridisation patterns shown are those of: (1) nick-translated total HSV-1 DNA, (2) *in vivo* labelled total IE mRNA, (3) IE cDNA prepared using total IE mRNA. The *Bam*HI k fragment used in this experiment spans the joint region from within b′ (in IR_L) to within c′ (in IR_S). The cuts made in *Bam*HI k by further digestion with *Sma*I (or its isoschizomer *Xma*I) and *Taq*I are indicated. The location of the 3′ portion of IE mRNA-3 and the approximate location of the 5′ portion of IE-mRNA-1 are shown

nuclease S1-resistant band of 2.1 kb was detected on both neutral and alkaline gels (data not shown). This placed the 3′ end of IE mRNA-3 within the *Sma*I a fragment. 3′-Labelled *Xma*I a (*Xma*I is an isoschizomer of *Sma*I which makes staggered cuts) yielded a single nuclease S1-resistant product of 240 bases (data not shown).

The location of the 3′ end of IE mRNA-3 was confirmed using *Taq*I d (Figure 3). 3′-Labelled *Taq*I d was strand-separated by electrophoresis on an 8% denaturing polyacrylamide gel, and hybridisations were performed using each DNA strand. Nuclease S1-resistant products, ranging in length from 81 to 85 bases, were formed by the faster migrating strand (Figure 4, tracks 3, 4, and 5). This DNA strand was also sequenced (Figure 4). The position of the 3′ end, shown in Figure 7, was determined by alignment of the nuclease S1-resistant bands alongside this sequence.

To confirm that the 81−85 base nuclease S1-resistant bands were generated by IE mRNA-3, the hybridisation was repeated with IE mRNA-3 which had been purified from a methyl-mercuric hydroxide agarose gel as described by Watson *et al.* (1979). Similar nuclease S1-resistant bands were again produced with the faster migrating *Taq*I d strand (Figure 4, track 6).

Location of the 5′ end

Previous Southern blot hybridisation (Watson *et al.*, 1979; Anderson *et al.*, 1980) had mapped IE mRNA-3 to *Bam*HI y. Therefore, nuclease S1 analysis was performed using this fragment and a number of sub-fragments generated by further restriction enzyme cleavage. Intact *Bam*HI y was protected completely from nuclease S1 digestion by IE mRNA, as were all the 5′-labelled *Xma*I, *Taq*I, *Pvu*II, and *Hinf*I sub-fragments of *Bam*HI y which were examined. The relevant maps and the hybridisation data for the 5′-labelled *Xma*I

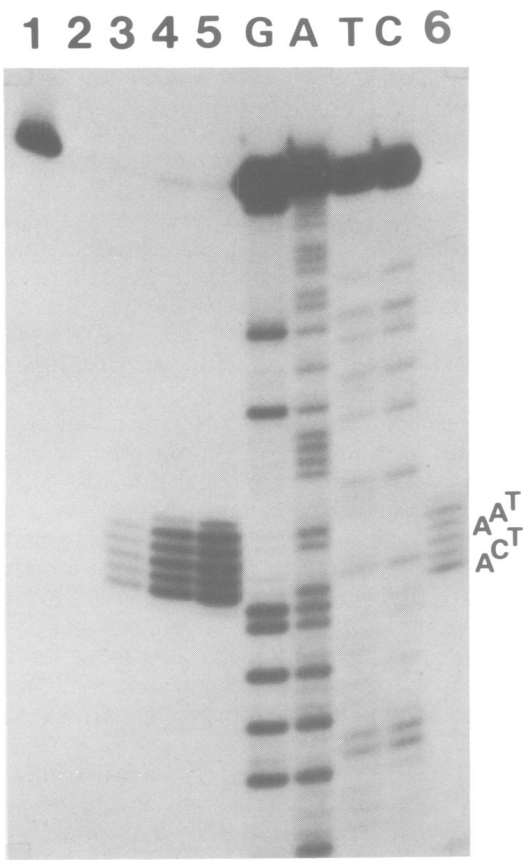

Fig. 4. Location of the 3′ end of IE mRNA-3. The *Taq*I d sub-fragment of *Bam*HI k (Figure 3) was 3′-labelled and strand separated. Portions of the faster migrating strand were sequenced by the method of Maxam and Gilbert (1980) or hybridised with the following samples: **1**, the unhybridised probe control; **2**, 20 μg of mock-infected cytoplasmic RNA; **3**, 5 μg of IE cytoplasmic RNA; **4**, 10 μg of IE cytoplasmic RNA; **5**, 15 μg of IE cytoplasmic RNA; **6**, IE mRNA-3 isolated from a methyl-mercuric hydroxide agarose gel (Bailey and Davidson, 1976). The nuclease S1-resistant material and sequence reaction products were electrophoresed on an 8% denaturing polyacrylamide gel. Sequencing of the labelled DNA was performed by A.J. Davison.

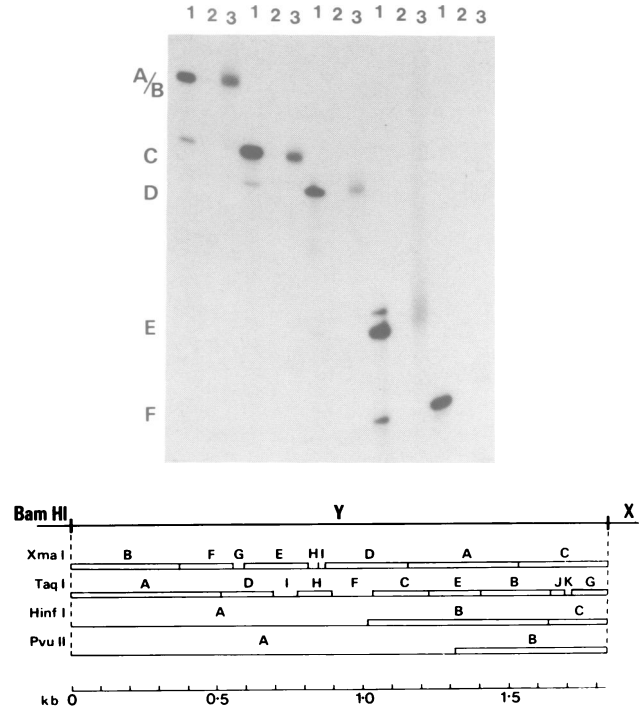

Fig. 5. IE mRNA sequences mapping in *Bam*HI y. Five size clases of 5′-labelled *Xma*I sub-fragments of *Bam*HI y were isolated from a non-denaturing polyacrylamide gel and hybridised with the following samples: **1**, 15 μg of IE cytoplasmic RNA; **2**, 20 μg of mock-infected cytoplasmic RNA; **3**, unhybridised probe. The nuclease S1-resistant material was electrophoresed on an 8% denaturing polyacrylamide gel. Similar analyses were performed using the other sub-fragments of *Bam*HI y indicated by double lines on the restriction enzyme maps for *Taq*I, *Hinf*I, and *Pvu*II. In all cases, the labelled DNA probe was totally protected from nuclease S1 digestion following hybridisation to IE cytoplasmic RNA.

sub-fragments of *Bam*HI y are shown in Figure 5. These results were consistent and indicated that IE mRNA-3 was unspliced within *Bam*HI y and that the 5′ end was located either at the very end of, or outside, this fragment. Due to the unusual structure of HSV DNA, there are two alternative neighbouring fragments here, *Bam*HI x (Figure 5) and *Bam*HI n (Figure 7), each spanning the short repeat region/U_S junction.

To locate precisely the 5′ end, the terminal *Eco*RI (150 bp), *Ava*II (260 bp), and *Taq*I (200 bp) fragments of *Bam*HI x which map adjacent to the *Bam*HI y/*Bam*HI x junction (Rixon and Clements, 1982), were isolated, 5′-labelled and strand-separated. For each fragment, only the slower migrating DNA strand formed nuclease S1-resistant bands with IE mRNA. The 25 – 30 base-long bands produced with a 5′-labelled *Taq*I fragment are shown in Figure 6. The slower migrating strand was sequenced and the position of the 5′ end on this DNA sequence is shown in Figure 7.

Discussion

Our mapping data, using intact HSV-1 DNA and overlapping restriction enzyme fragments, indicate that IE mRNA-3 is unspliced. In this respect, it resembles the majority of HSV-1 mRNAs so far examined (Wagner *et al.*, 1981; Anderson *et al.*, 1981; Costa *et al.*, 1981; Frink *et al.*, 1981; McLauchlan and Clements, 1982). Two HSV-1 IE mRNAs are known to be spliced; these have a common 5′ portion containing an intron which is located 5′ to their translational initiation codons (Watson and Vande Woude, 1982; Rixon and Clements, 1982). The mRNAs from a single late trancriptional unit also have been reported to be spliced (Frink *et al.*, 1981).

Examination of the genomic DNA sequences located at the 5′ end of IE mRNA-3 reveals sequences resembling consensus signals involved in the initiation of transcription. The 5′ end is located at a sequence (TCGTCCA) which resembles the consensus cap site (Py-CATTCPu) (Busslinger *et al.*, 1980); a potential 'TATA' box (Gannon *et al.*, 1979) is located 26 – 30 bp upstream from the 5′ end and a possible GGCCC pentameric sequence (consensus GATCC) (Busslinger *et al.*, 1980) is located 38 – 42 bp upstream from the 5′ end.

The presence of these sequences in the appropriate locations strongly supports the belief that the 5′ end of this mRNA has been correctly identified. Our map position for the 5′ end is also supported by the results of Mackem and Roizman (1980). Using labelled mRNA as a probe for Southern blot hybridisations, they localised the 5′ end of IE mRNA-3 within *Bam*HI x or n, close to the junction with *Bam*HI y.

The 5′ end of IE mRNA-3 is separated by ~700 bp from the 5′ end of IE mRNA-4 and -5 (Murchie and McGeoch,

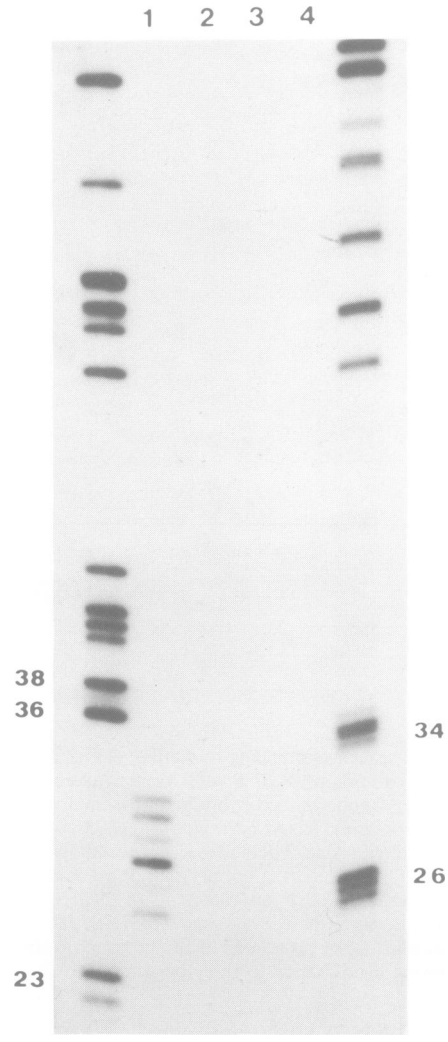

Fig. 6. Location of the 5′ end of IE mRNA-3. The terminal 200-bp *Taq*I sub-fragment of *Bam*HI x was isolated, 5′-labelled and strand-separated. The slower migrating strand was hybridised to: **1**, 15 μg of IE cytoplasmic RNA; **2**, 20 μg of mock-infected cytoplasmic RNA. The faster migrating strand was hybridised to: **3**, 15 μg of IE cytoplasmic RNA; **4**, 20 μg of mock-infected cytoplasmic RNA. The nuclease S1-resistant material was electrophoresed on a 8% denaturing polyacrylamide gel. The size standard on the right-hand track was *Hpa*II-digested pBR322 DNA and that on the left-hand track was 3′-labelled *Hinf*I fragments of pBR322 DNA, after digestion with *Hæ*III.

1982). The intervening DNA sequence does not appear to encode any additional mRNAs. However, a potential origin for DNA replication has been mapped to this region (Stow, 1982).

An 'AATAAA' polyadenylation signal (Proudfoot and Brownlee, 1976) is located 17 – 22 bp upstream of the 3′ end of IE mRNA-3. This 3′ end lies ~800 bp from the 'a' sequence which defines the joint between the long repeat and short repeat genome regions. This distance varies among different DNA molecules. The DNA sequence for this region includes two families of tandem reiterations (Davison and Wilkie, 1981) and the size variability among DNA molecules from this region appears to be a function of different copy numbers of these tandem reiterations.

Evidence has been obtained, by Northern blot analysis of IE, early, and late mRNA, suggesting that no additional mRNAs map at the region specifying IE mRNA-3 (F.J.Rixon, unpublished data). Hence, IE mRNA-3 is not a component of an overlapping family of mRNAs, such as have been found at other regions of the HSV-1 genome which are expressed at early and late times (Anderson *et al.*, 1981; Costa *et al.*, 1981; Frink *et al.*, 1981; McLauchlan and Clements, 1981). Furthermore, the mRNAs mapping in the short repeat regions are bounded by considerably longer untranscribed regions than are normal in the other DNA viruses examined to date (Ziff, 1980).

As IE mRNA-3 maps with TR_S and IR_S the HSV-1 genome may be thought of as effectively diploid for this gene. This is a feature shared with IE mRNA-1 which maps entirely within TR_L and IR_L (Watson *et al.*, 1979; Anderson *et al.*, 1980). Analysis of HSV-1/HSV-2 intertypic recombinants which were heterotypic for TR_L/IR_L indicated that both copies of the gene specifying IE mRNA-1 were expressed (Davison *et al.*, 1981). It is not clear whether having two copies of these particular genes confers any advantage on the virus. Possession of unique DNA regions and repeated DNA regions is a feature of the organisation of all investigated herpesvirus genomes, suggesting a special role for this type of genome organisation in the herpesvirus life cycle. All clinical isolates of HSV-1 and HSV-2 analysed to date have been diploid for both repeat regions. However, an intertypic recombinant of HSV-1 and HSV-2 lacking most of the TR_L region grew normally in tissue culture cells (Davison *et al.*, 1981). Furthermore, analysis of human cytomegalovirus

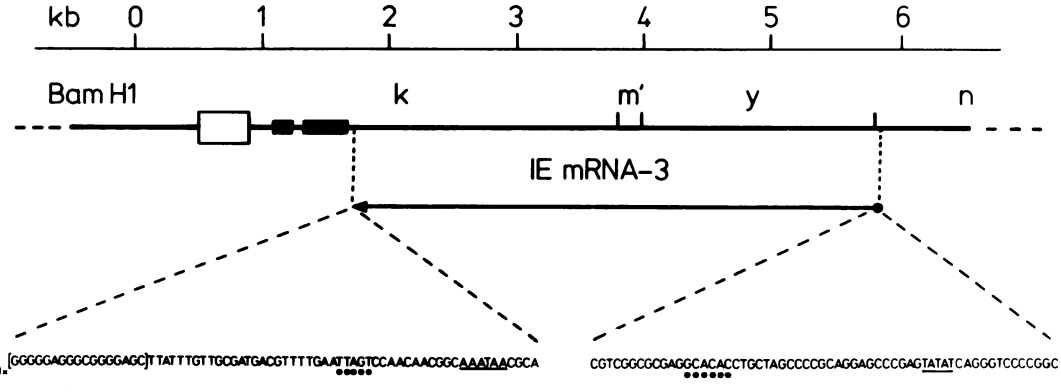

Fig. 7. Summary diagram showing the map location, orientation, structure, and nucleotide sequences encoding the 5′ and 3′ ends of IE mRNA-3. The open box represents the 'a' sequence which defines the long region/short region joint. The two solid boxes represent the regions of tandemly reiterated sequence which lie between the 3′ end of IE mRNA-3 and the 'a' sequence (Davison and Wilkie, 1981). The 5′ and 3′ ends of IE mRNA-3 map within the regions indicated by asterisks. The 'TATA' and 'AATAAA' consensus signals, upstream of the 5′ and 3′ ends respectively, are underlined. The sequence encoding the 5′ end is reproduced from Murchie and McGeoch (1982) and that encoding the 3′ is reproduced from Davison and Wilkie (1981).

(CMV) has shown that the repeated DNA regions do not necessarily encode IE genes (De Marchi, 1981), although this has been reported for some human CMV strains (Wathen *et al.*, 1981). Therefore, while the presence of repeated DNA sequences may be of fundamental importance for herpesvirus growth, the expression of two copies of specific IE genes appears not to be essential. This being the case, the presence of two copies of these particular HSV-1 IE genes may be a consequence of their genome location rather than indicative of a requirement for two gene copies in the infectious cycle.

Materials and methods

Cells and virus

Baby hamster kidney 21 (C13) cells were grown as monolayers in rotating 80 oz bottles. For the production of IE mRNA, cell monolayers were infected with HSV-1 (Glasgow strain 17) at a multiplicity of infection of 50 p.f.u./cell. The cell monolayers were pretreated and maintained in medium containing cycloheximide (200 μg/ml) as described previously (Clements *et al.*, 1977).

Preparation of cytoplasmic RNA

Cytoplasmic RNA was prepared using the procedure of Kumar and Lindberg (1972).

Enzymes

All enzymes were obtained from Bethesda Research Laboratories or New England Biolabs, with the exception of T4 polynucleotide kinase (P-L Biochemicals) and nuclease S1 (Boehringer Corporation Limited). The digestion procedures used were those recommended by the manufacturers.

Cloning procedures

Fragments of HSV-1 DNA, generated by using restriction endonucleases, were cloned into pAT 153 and grown in *Escherichia coli* K12 HB101. The procedures used for cloning and isolation of cloned virus DNA were those described by Davison and Wilkie (1981).

Purification and end-labelling of DNA fragments

Purification of DNA fragments from agarose or polyacrylamide gels, and labelling of the 5′ and 3′ ends was carried out as described by McLauchlan and Clements (1982).

Structural analysis of mRNAs

Structural analysis of mRNAs was performed using the nuclease S1 digestion procedure of Berk and Sharp (1978) which was modified by using either 5′ or 3′ end-labelled DNA probes in addition to *in vivo* labelled DNA.

Labelled DNA (<1 μg) was co-precipitated with known amounts of cytoplasmic RNA from infected or mock-infected cells. The DNA/RNA pellet was resuspended in 20 μl of 90% (v/v) formamide (deionised with Amberlite monobed resin MB-1), 400 mM NaCl, 40 mM PIPES, pH 6.8, 1 mM EDTA. This mixture was heated to 90°C for 3 min then incubated at 57°C or 57.5°C (when single-stranded DNA probes were used, the hybridisation temperature was reduced to 37°C) for either 5 h or 16 h. Prior to nuclease treatment, the hybridisation mixtures were rapidly quenched on ice.

Nuclease S1 digestion was performed at 37°C for 1 h in 200 μl of 250 mM NaCl, 30 mM NaOAc, pH 4.5, 1 mM $ZnSO_4$ with 4000 units of nuclease S1. The nuclease S1-digested hybrids were extracted with phenol/chloroform, precipitated with ethanol and analysed by gel electrophoresis.

Analysis of small hybrids on denaturing gels often revealed microheterogeneity of the bands, which is due to the imprecise cleavage at hybrids ends by nuclease S1.

Gel electrophoresis

Electrophoresis of samples was performed, either on non-denaturing 1.5% (w/v) agarose gels or on two-dimensional gels as described by Rixon and Clements (1982).

Denaturing polyacrylamide gels, essentially as described by Maxam and Gilbert (1980), were run in 90 mM Tris, 90 mM boric acid, pH 8.3, 1 mM EDTA. Samples were dissolved in deionised formamide and denatured at 90°C for 2 min before loading. Electrophoresis was carried out at room temperature for 3–6 h at 40 watts. The radiolabelled bands were visualised by autoradiography.

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