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A 3' co-terminus of two early herpes simplex virus type 1 mRNAs

John McLauchlan and J.Barklie Clements

Institute of Virology, University of Glasgow, Glasgow G11 5JR, UK

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ABSTRACT

A 3' co-terminus of two early herpes simplex virus type 1 mRNAs has been identified using the nuclease-S1 mapping procedure with cloned virus DNA probes. These mRNAs (5.0 kb and 1.2 kb), located within the genome region 0.56-0.60, are unspliced and are transcribed rightwards on the prototype genome orientation. The position of their 3' ends has been located on the virus DNA sequence and lies downstream from the polyadenylation signal 5'-AATAAA-3'. This hexanucleotide sequence also was present in the complementary DNA strand and was shown to be the polyadenylation signal for a leftwards-transcribed late mRNA. The abundance within the cytoplasm of the 5.0 kb and 1.2 kb mRNAs was investigated. Results indicated that these mRNAs were regulated in concert. It is suggested that sequences at the 3' co-terminus may be involved in their regulation.

INTRODUCTION

A feature of studies on the organisation of papovavirus and adenovirus transcription units has been the identification of mRNA families which are 5' and 3' co-terminal (reviewed by Ziff, 1). This arrangement allows the use of common regulatory signals involved in the initiation of transcription and in polyadenylation of RNA. Within such transcription units, the different mRNAs are generated by differential splicing.

We have characterised the 5' termini of two early herpes simplex virus type 1 (HSV-1) mRNAs (2) which map in the HindIII k/BamHI o region of the virus genome. The 5' portions of these mRNAs are unspliced and extend 4.2 kb and 0.39 kb to the left of the HindIII cleavage site at co-ordinate 0.586 (Figure 1). Regulatory signals involved in transcription initiation of the smaller (1.2 kb) mRNA are located within the larger (5.0 kb), overlapping mRNA. Hybrid selection experiments have indicated that the 5.0 kb mRNA encodes a polypeptide of 136,000 molecular weight while a 38,000 polypeptide appears to be specified by the 1.2 kb mRNA (3).

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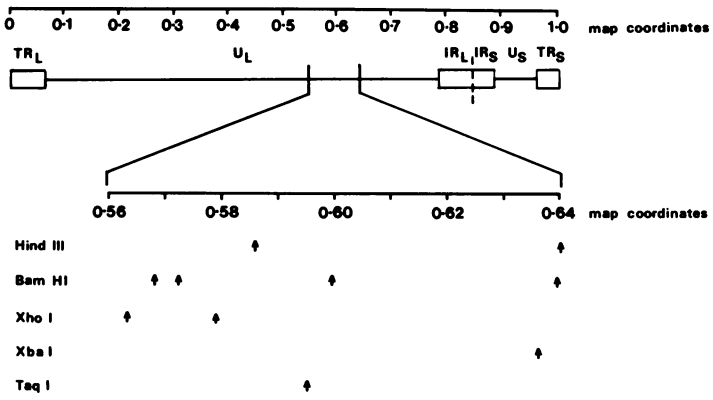

Fig. 1 Restriction endonuclease cleavage maps of HSV-1 strain 17 DNA at the genome region located between co-ordinates 0.56-0.64. The prototype genome orientation is shown and only one of several Taq I cleavage sites is indicated.

Anderson et al. (3), by nuclease-S1 digestion of DNA/RNA hybrids formed with uniformly-labelled DNA, have suggested that these mRNAs are 3' co-terminal.

Here we locate the 3' termini of these mRNAs using 3'-labelled DNA probes to precisely position the ends. Genomic DNA at the 3' termini was sequenced to examine for signals that may be involved in polyadenylation of RNA. The appearance and abundance within the cytoplasm of the 5.0 kb and 1.2 kb mRNAs was investigated using 5'-labelled DNA probes.

MATERIALS AND METHODS

Cells and virus Baby hamster kidney 21 (C13) cells were grown as monolayers in 800 ml plastic tissue culture flasks (4). For the production of early and late classes of RNA, cell monolayers were infected with HSV-1 (Glasgow strain 17) at a multiplicity of infection of 10 p.f.u./cell. The multiplicity of infection was increased to 50 p.f.u./cell to produce IE RNA and cycloheximide-released RNA. As appropriate, cell monolayers were pretreated and maintained in medium containing cycloheximide as described previously (4). For isolation of cycloheximide-released RNA, the cycloheximide was removed by washing the cells three times with cycloheximide-free medium at 37°C. Infection was then continued for 1 h in cycloheximide-free medium at 37°C after which the RNA was isolated.

Cell fractionation and isolation of RNA Cytoplasmic cell fractions were

prepared and RNA was isolated as previously described (5).

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). DNA was digested with restriction endonucleases at 37°C in 50-200 µl 0.006 M Tris-HCl, pH 7.5, 0.006 M $MgCl_2$ and 0.006 M 2-mercaptoethanol.

Cloning Procedures Fragments of HSV-1 DNA generated using restriction endonuclease were cloned within the Institute of Virology under Category I containment (U.K. Genetic Manipulation Advisory Group). The host bacterium was E.coli K12 HB101 and the cloning vector was pAT 153. Isolation of cloned virus DNA was as described by Davison and Wilkie (6).

Purification of DNA fragments DNA fragments were purified from agarose gels by hydroxylapatite column chromatography (7).

Fragments were eluted from polyacrylamide gels by incubating gel slices in 500 mM NH_4OAc, 0.1% (w/v) SDS at 42°C overnight. Acrylamide was removed by filtration through glass wool and the DNA in the eluate was precipitated with ethanol at -20°C.

End-labelling of DNA DNA fragments were 5'-labelled by a transfer reaction. Initially, the DNA was digested with 2 units of bacterial alkaline phosphatase/pmole of fragment ends in 10 mM Tris-HCl, pH 8.0 for 1 h at 60°C. The phosphatase was removed by phenol/chloroform extraction and the DNA was precipitated with ethanol. 5'-labelling was performed in 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT with 5-10 units of T_4 polynucleotide kinase and 10 µCi of (γ-^{32}P) ATP (specific activity 5000 Ci/mMol, Radiochemical Centre, Amersham) for 1 h at 37°C.

Fragments containing recessed 3' ends were 3'-labelled by a 'filling in' reaction. Reactions were incubated in 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1 mM DTT with 5 units of large fragment DNA polymerase I and 5-10 µCi of the appropriate (α-^{32}P) deoxyribonucleoside triphosphate (specific activities 2000-3000 Ci/mmol from the Radiochemical Centre, Amersham).

In the experiments described here, the DNA fragments, either 5'- or 3'-labelled at both ends normally were redigested with a second restriction endonuclease in order to generate fragments with uniquely-labelled ends.

Structural analysis of mRNAs This was performed using the nuclease-S1 mapping procedures essentially as described by Berk and Sharp (8). An extension of this procedure (9) was to use either 5'- or 3'-labelled DNA probes instead of uniformly-labelled DNA. The 5' end of an mRNA or a splice acceptor site can be located with 5'-labelled DNA probes while 3' ends and

splice donor sites are positioned with 3'-labelled probes. Splices can be detected by a comparison of nuclease-S1-resistant material on non-denaturing and denaturing gels. A reduction in size on a denaturing gel denotes the presence of a splice in the RNA while similar sizes on the two gel systems indicates that the RNA is unspliced within the DNA fragment used as a probe.

Either 5'- or 3'-labelled DNA (less than 1 µg) was co-precipitated with 50 µg of cytoplasmic RNA. The DNA/RNA pellet was resuspended in 20 µl of 90% (v/v) formamide (deionised with Amberlite monobed resin MB-1), 0.4 M NaCl, 40 mM PIPES, pH 6.8, 1 mM EDTA. This mixture was heated to 80-90°C for 3 min then incubated at 57°C or 57.5°C for either 5 h or 16 h. Prior to nuclease treatment, the hybridisation mixtures were rapidly quenched on ice. Nuclease-S1 treatment of hybrids was performed at 37°C for 1.5h in 200µl of 0.25M NaCl, 30mM NaOAc, pH 4.5, 1 mM $ZnSO_4$ with 4000 units of nuclease-S1. The nuclease-digested hybrids were extracted with phenol/chloroform then precipitated with ethanol. The nuclease-S1-resistant material was examined by gel electrophoresis.

Gel Electrophoresis Samples were electrophoresed either on non-denaturing 1.5% (w/v) agarose gels in a buffer containing 90 mM Tris, 90 mM boric acid, pH 8.3, 1 mM EDTA or on alkaline 1.5% (w/v) agarose gels in 30 mM NaOH, 2 mM EDTA. Electrophoresis was carried out at room temperature for 16 h at 13 mA. The gels were then dried down and the bands visualised by autoradiography at -70°C using Kodak X-Omat-S film. Denaturing and sequencing polyacrylamide gels, essentially as described by Maxam and Gilbert (10), were run in 90 mM Tris, 90 mM boric acid, pH 8.3, 1 mM EDTA and the gels contained 9 M urea. 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 detected by autoradiography.

DNA sequencing DNA sequences were determined by chemical degradation (10) of 3'-labelled DNA fragments.

RESULTS

3' termini of rightwards-transcribed mRNAs which map in HindIII l The 5' termini of the 5.0 kb and 1.2 kb mRNAs are located 4.2 kb and 0.39 kb respectively within HindIII k (2) across the HindIII cleavage site at co-ordinate 0.586 (Figure 1). Fragment HindIII l (Figure 1, co-ordinates 0.586-0.64) was 3'-labelled at both HindIII sites then cleaved with XbaI. The larger, uniquely 3'-labelled fragment was hybridised to RNA samples from infected and mock-

infected cells. Following nuclease-S1 treatment, samples were electrophoresed on 1.5% (w/v) neutral and alkaline agarose gels (Figure 2).

A single protected band of 770 bp was detected with the cycloheximide-released, infected cell RNA and the band was not present in the mock-infected or IE RNA samples. The 770 bp size was similar on neutral and alkaline gels indicating that this 3' portion of the two mRNAs was unspliced.

A more precise location of the 3' terminus was obtained using a Taq 1 cleavage site at co-ordinate 0.595 (Figure 1). A DNA probe 3'-labelled at this Taq 1 site was hybridised to different RNA samples. Following nuclease-S1 treatment and separation on a denaturing polyacrylamide gel, a single protected fragment of 98 bp was detected (Figure 3). Similar amounts of this band were observed with the 3 h and cycloheximide-released RNAs (Figure 3A, tracks 1 and 2) and a small amount was observed with IE RNA (Figure 3A, track 3).

Nucleotide sequence at the 3' co-terminus of the 5.0 and 1.2 kb mRNAs

The sequence was determined by the chemical method for both DNA strands using cloned virus DNA fragments which were uniquely 3'-labelled at restriction endonuclease cleavage sites. A sequencing gel of the DNA fragment labelled at the Taq 1 site (Figure 1, co-ordinate 0.595) which formed the 98 bp hybrid with infected cell RNA is shown in Figure 3B. The sequence on the gel is of the DNA strand complementary to the 5.0 kb and 1.2 kb mRNAs. The S1-

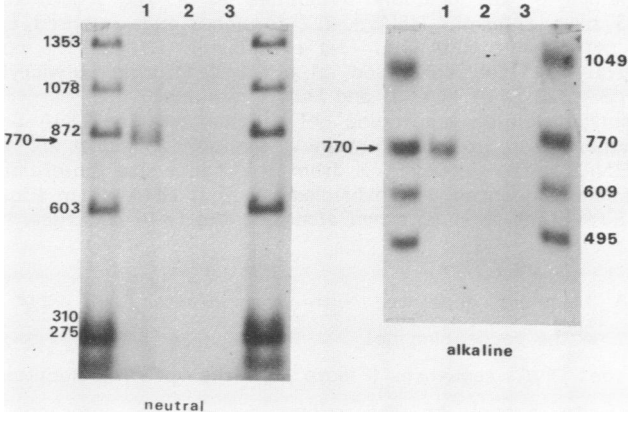

Fig. 2 Analysis of the 3' co-terminus of the 5.0 kb and 1.2 kb mRNAs. The DNA probe was uniquely 3'-labelled at a HindIII site (co-ordinate 0.586) and was hybridised to various RNA samples. 1. Cycloheximide-released cytoplasmic RNA. 2. IE cytoplasmic RNA. 3. MI cytoplasmic RNA. The nuclease-S1-resistant material was electrophoresed on a 1.5% (w/v) neutral agarose gel together with ØX174 DNA HaeIII markers, and on a 1.5% (w/v) alkaline agarose gel together with ØX174 DNA Hinc II markers.

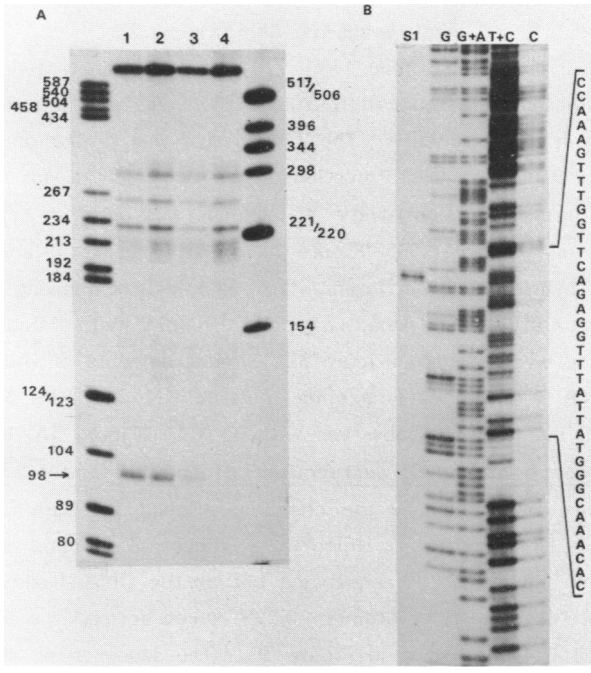

Fig. 3 (A) Precise map location of the 3' co-terminus. To generate the DNA probe, BamHI o was uniquely 3'-labelled at a Taq I site (co-ordinate 0.595). 1. 3 h cytoplasmic RNA. 2. Cycloheximide-released cytoplasmic RNA. 3. IE cytoplasmic RNA. 4. MI cytoplasmic RNA. The nuclease-S1-resistant material was electrophoresed on a 6% denaturing polyacrylamide gel together with pBR 322 DNA Hae III and Hinf I markers.
(B) An 8% polyacrylamide sequencing gel which shows the DNA sequence of the strand complementary to that containing the poly(A) signal for the 5.0 kb and 1.2 kb mRNAs. The sequence is from the Taq I site (co-ordinate 0.595). The DNA fragment sequenced was hybridised to 3 h RNA as in Fig. 3(A) and the nuclease-S1-resistant band is shown alongside the DNA sequence.

protected DNA fragment generated with 3 h infected cell RNA also was electrophoresed on the sequencing gel.

From the DNA sequence (Figure 4), the precise location of the polyadenylation site cannot be determined due to the presence of three adenosine residues located at positions 95-97. The hexanucleotide sequence 5'-AATAAA-3', which appears to function as a polyadenylation signal for eukaryotic mRNAs, is located at positions 79-84, about 10 bp from the polyadenylation site of the co-terminal mRNAs. This hexanucleotide sequence also is found in the complementary strand at positions 210-215; potentially this

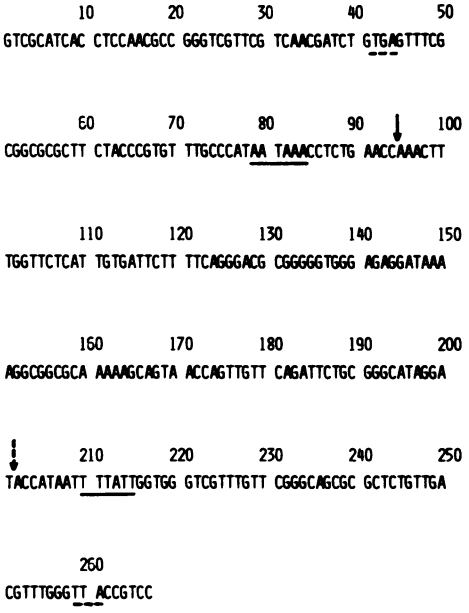

Fig. 4 Nucleotide sequence at the 3' co-terminus. The poly(A) signal for the two rightwards-transcribed mRNAs is underlined as is the poly(A) signal for a leftwards-transcribed mRNA. The 3' end of the 5.0 kb and 1.2 kb mRNAs and the 3' end of a leftwards-transcribed mRNA are arrowed. Translational stop codons, located within the transcribed region, are indicated by a dotted line.

could serve as a poly(A) signal for a leftwards-transcribed mRNA.

3' terminus of a leftwards-transcribed mRNA which maps in HindIII 1 To determine if the polyadenylation signal in the complementary strand to that containing the polyadenylation signal for the 5.0 kb and 1.2 kb mRNAs was associated with an mRNA, 3'-labelled BamHI o (Figure 1, co-ordinates 0.572-0.599) was cleaved with HindIII. The smaller fragment, uniquely 3'-labelled at co-ordinate 0.599, was hybridised to RNA isolated from infected cells at different times post-infection and to RNA from mock-infected cells.

After nuclease-S1 treatment and electrophoresis on denaturing gels, a single band of 528 nucleotides was observed (Figure 5) only with RNA isolated from cells at 6 h post-infection. The poly(A) site of this leftwards-transcribed mRNA is shown on the DNA sequence at position 196 (Figure 4); this location is determined to an accuracy of 4-5 nucleotides.

Temporal regulation of the 5.0 kb and 1.2 kb mRNAs The appearance and abundance within the cytoplasm of the 5.0 kb and 1.2 kb mRNAs was investigated using two DNA probes which allowed detection of the individual mRNAs. The 5.0 kb mRNA was examined using an XhoI DNA fragment 5'-labelled at co-ordinate 0.562 (Figure 1) and a fragment 5'-labelled at a HindIII site (Figure 1, co-ordinate 0.568) was used to detect the 1.2 kb mRNA.

Similar amounts of cytoplasmic RNA (50 µg) isolated at various times

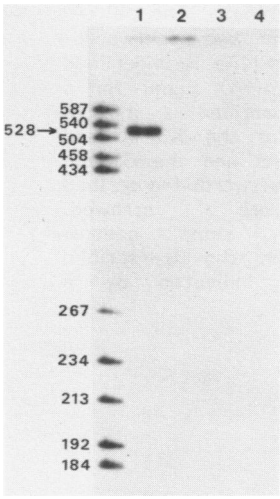

Fig. 5 Detection of a leftwards-transcribed mRNA. The DNA probe was 3'-labelled at a BamHI site (co-ordinate 0.599). 1. 6 h cytoplasmic RNA. 2. 3 h cytoplasmic RNA. 3. Cycloheximide-released cytoplasmic RNA. 4. MI cytoplasmic RNA. The nuclease-S1-resistant material was electrophoresed on a 6% denaturing polyacrylamide gel together with pBR 322 DNA HaeIII markers.

post-infection were hybridised to these DNA probes, and following nuclease-S1 treatment the samples were electrophoresed on denaturing polyacrylamide gels.

A fragment of 197 nucleotides was protected by the 5.0 kb mRNA (Figure 6A) while the 1.2 kb mRNA protected a fragment of 390 nucleotides (Figure 6B). The maximum amount of each mRNA was detected in the 3 h post-infection sample (Figure 6, track 3), followed by a decline in 6 h RNA, and little of both mRNAs was observed in RNA isolated at 16 h post-infection. A small amount of both mRNAs was detected in the IE mRNA sample.

DISCUSSION

The 5.0 kb and 1.2 kb mRNAs are transcribed rightwards on the prototype genome orientation (Figure 7) and the 5' portions are unspliced within HindIII k (2). Here, we have detected a single protected DNA band using DNA probes 3'-labelled at a HindIII cleavage site (Figure 1, co-ordinate 0.586) and at a Taq 1 cleavage site (Figure 1, co-ordinate 0.595). The sizes of the protected fragments were similar on neutral and alkaline gels.

The data demonstrate that the 3' portion of the two mRNAs is unspliced and that the mRNAs are 3' co-terminal. This confirms the suggestion of Anderson et al. (3); these authors have described a third 7.0 kb mRNA which appeared to be 3' co-terminal with the 5.0 kb and 1.2 kb mRNAs. We did not detect a 7.0 kb mRNA which mapped at this locus.

The hexanucleotide sequence 5'-AAUAAA-3' (11) or a related

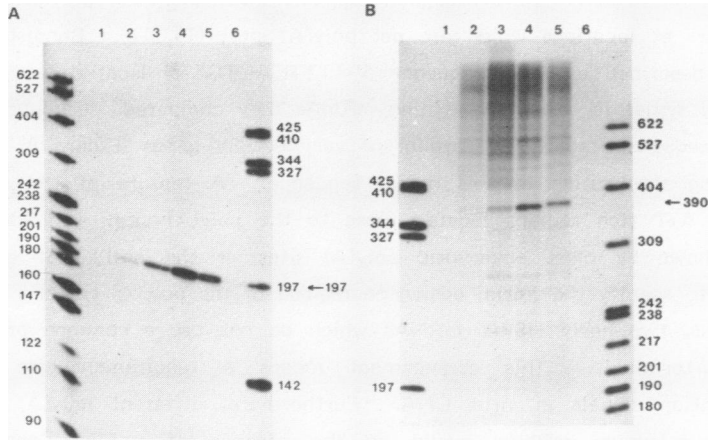

Fig. 6 Temporal regulation of the 5.0 kb and 1.2 kb mRNAs.
(A) The 5.0 kb mRNA was investigated by 5'-labelling HindIII k at a XhoI site (coordinate 0.562)
(B) The 1.2 kb mRNA was examined by 5'-labelling BamHI o at a HindIII site (co-ordinate 0.586). 1. MI cytoplasmic RNA. 2. IE cytoplasmic RNA. 3. Cycloheximide-released cytoplasmic RNA. 4. 3 h cytoplasmic RNA. 5. 6 h cytoplasmic RNA. 6. 16 h cytoplasmic RNA. The nuclease-S1-resistant material was electrophoresed on 6% denaturing polyacrylamde gels together with pBR 322 DNA HpaII markers. 3'-labelled HinfI fragments of pBR 322 DNA after digestion with HaeIII, also were used as markers.

sequence (12, 13) is located 10-20 nucleotides proximal to the 3' terminal poly(A) of polyadenylated mRNAs. This sequence was located close to the 3' termini of the HSV-1 mRNAs examined here. The signal is involved in polyadenylation as a deletion within the sequence prevents poly(A) addition, and tandem duplications generate two classes of late SV40 mRNAs which result from the use of both signals (14).

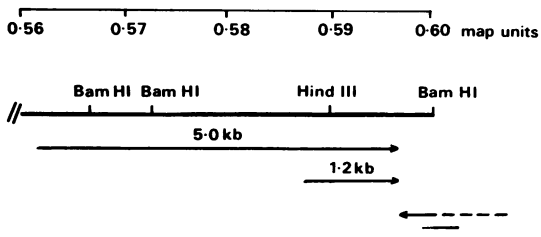

Fig. 7 Summary of the genome map locations and orientations of the HSV-1 mRNAs mapping between co-ordinates 0.56-0.60.

The sequence AAUAAA alone appears insufficient for polyadenylation as it occurs at locations which are not poly(A) sites (15, 16). Benoist et al. (17) have described a model sequence 5'-TTTTCACTGC-3' located just beyond the poly(A) signal for five of the nine mRNAs they compared. Comparison of the DNA sequences at the 3' termini of various HSV genes (Figure 8) reveals few striking similarities to this model sequence. A feature of the HSV 3' termini is A+T rich regions located close to the poly(A) site. An additional feature, shown by most eukaryotic poly(A) sites, is the ability of genomic sequences to specify the initial adenosine residue of the poly(A) tract.

Thus, two early HSV-1 mRNAs which do not use a common promoter are 3' co-terminal. This arrangement means a requirement for fewer polyadenylation signals in virus DNA. Furthermore, different poly(A) signals used by overlapping mRNAs would, in the absence of splicing, require a mechanism whereby the poly(A) signal for the appropriate mRNA was selected. The HSV IE genes so far analysed do not have 3' co-termini however these transcription units each appear to specify only a single mRNA (21).

Maximum amounts of the 5.0 kb and 1.2 kb mRNAs within the cytoplasm were obtained with early (3 h) RNA, and little of either mRNA was detected in RNA isolated at 6 h post-infection. Small amounts of these mRNAs were detected in IE RNA and this may result from residual protein synthesis occurring in the presence of cylcoheximide.

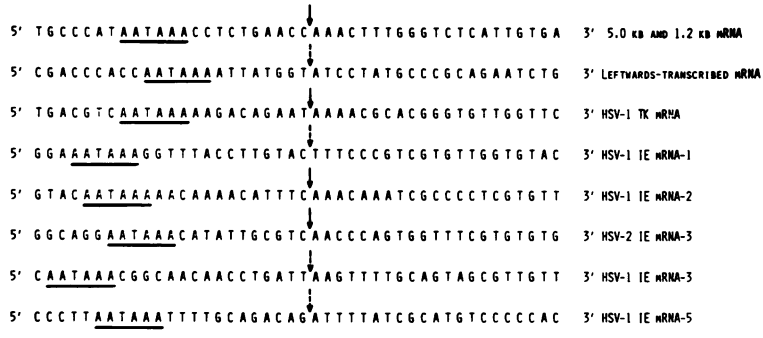

Fig. 8 Comparison of the 3' terminal sequences of various HSV genes. Locations of 3' ends are arrowed. Dotted arrows represent uncertainty as to the precise location of the terminal nucleotide. The poly(A) signal 5'-AATAAA-3' of each mRNA is underlined. Sources for the 3' terminal sequences were as follows; HSV-1 TK mRNA (18); HSV-1 IE mRNA-1 and -5 (F. Rixon, personal communication); HSV-1 IE mRNA-2 and HSV-2 IE mRNA-3 (19); HSV-1 IE mRNA-3 (6). The numbering of HSV IE mRNAs is as described by Clements et al. (20).

As the 5.0 kb ad 1.2 kb mRNAs have a similar pattern of temporal regulation this suggests that they may be regulated by a common control mechanism. Such control could be at the level of transcription initiation but termination of transcription also is a possibility. This type of control is exerted at early times to prevent expression of sequences from the L4 and L5 co-terminal families of the late adenovirus 2 transcription unit. Also, analysis of primary transcripts within the nucleus at early times has shown that L1 mRNAs are at a higher concentration than L2 mRNAs whereas at later times the opposite situation occurs (22).

A 3' terminus of one or more mRNAs which are transcribed leftwards (Figure 7) from the complementary DNA strand to that specifying the 5.0 kb and 1.2 kb mRNAs has been mapped. In contrast to the 5.0 kb and 1.2 kb mRNAs, the leftwards-transcribed mRNA was detected only with 6 h RNA which indicates that it is synthesised at late times. Approximately 100 nucleotides separates the two 3' termini (Figure 4). This is unlike the adenovirus 2 'strand switch' points described so far in which the 3' ends of mRNAs overlap for a short distance (23, 24). as do early and late SV40 mRNAs (25).

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