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Orientation of herpes simplex virus type 1 immediate early mRNA's

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ABSTRACT

We have determined the orientation of 4 immediate early (IE) mRNA's on the herpes simplex virus type 1 genome by mapping cDNA's complementary to the 3'-termini of messages. These IE mRNA's are transcribed by a pre-existing cell RNA polymerase, and we propose a model which allows their synthesis from a circular template using a single virus promoter region. The promoter region, which is located in the two repetitive DNA regions which flank the short unique region of the virus genome, may serve to initiate bidirectional transcription of these IE mRNA's.

INTRODUCTION

Transcripts synthesised in cells infected with herpes simplex virus type 1 (HSV-1) at early and late times post-infection (before and after the onset of virus DNA replication) hybridise to DNA fragments which map throughout the virus genome (1). In contrast, those transcripts which accumulate when protein synthesis is inhibited with cycloheximide, hybridise only to certain regions of the genome (1, 2). This restricted set of RNA sequences is transcribed by a pre-existing, α-amanitin-sensitive RNA polymerase (3), and has been termed immediate early (IE) RNA. IE RNA's also have been shown to accumulate in cells infected at non-permissive temperature with certain temperature sensitive mutants of HSV-1 (4).

The polypeptides specified by IE mRNA form a sub-set of the virus polypeptides made during productive infection (5, 6) and analyses of virus genomes and polypeptides expressed by HSV-1/HSV-2 intertypic recombinants have allowed the mapping of several type-specific IE polypeptides (6). These polypeptide locations have been confirmed by RNA mapping and in vitro

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translation of separated IE mRNA's (7).

In our laboratory, IE polypeptides are identified by their apparent molecular weights and these include HSV-1 IE polypeptides designated as Vmw 175, 110, 68, 63 and 12 (6, 7). These polypeptides are phosphorylated, preferentially accumulate in the nucleus (8) and have DNA binding properties in vitro (9). The IE polypeptides include the α-polypeptide class as defined by Honess & Roizman (5) synthesis of which is required for subsequent appearance of later polypeptides.

The evidence suggests that transcription of the HSV genome is subject to temporal regulation, and indicates that IE mRNA's code for one or more polypeptides which regulate a major event in virus transcription namely, the transition from IE to early RNA synthesis.

We have previously mapped separated, polyadenylated IE mRNA's on the HSV-1 genome and also used these mRNA's to direct protein synthesis in vitro (7). Here, we report on experiments designed to determine the orientation of IE mRNA's on HSV-1 DNA, by mapping complementary DNA (cDNA) prepared against the 3'-termini of IE mRNA's, and present a model which allows the various IE mRNA's to be synthesised using a single virus promoter region.

MATERIALS AND METHODS

Cells and Virus

Baby hamster kidney (BHK21-C13) cells were grown to confluence as monolayers (2-3 x 10^8 cells) in 2 litre roller bottles, and infected with HSV-1 (Glasgow strain 17) at a multiplicity of 50-100 pfu/cell as described previously (1). For purification of non-radioactive RNA, cells were pretreated with 200 µg/ml cycloheximide for 1 hr prior to virus infection. Virus infection and subsequent maintenance of monolayers was carried out at 37°C in the presence of 200 µg/ml cycloheximide for 6 hr post-infection. For preparation of labelled RNA, cell monolayers were infected as above and then labelled with 7-10 mCi (^{32}P)-orthophosphate from 0-6 hr post-infection.

Cell fractionation and isolation of RNA

Cytoplasmic RNA was prepared by lysing cells with 0.5% NP40, removing nuclei by low-speed centrifugation, and phenol/chloroform extraction of the cytoplasmic fraction (10). Polyadenylated cytoplasmic RNA was selected as described previously (7).

In vitro labelling of RNA

Non-radioactive HSV-1 IE polyadenylated RNA was labelled with (γ-^{32}P)-ATP using polynucleotide kinase. To provide 5'-OH termini for the labelling reaction, RNA was partially degraded by heating to 68°C for 20 min. in 10 mM Tris-HCl pH 8.8, and the degraded RNA was ethanol precipitated prior to labelling. Terminal labelling reactions contained 1-5 µg RNA, 40 µCi (γ-^{32}P)-ATP (Radiochemical Centre, Amersham; specific activity, 3000 Ci/mmol) 50 mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 10 mM DTT and 3.5 units of polynucleotide kinase (PL Biochemicals) in a total volume of 25 µl. Mixtures were incubated at 37°C for 2 hr and reactions terminated by addition of SDS and EDTA to 1% and 20 mM respectively. After addition of 50 µg carrier yeast RNA, the labelled RNA was chromatographed on Sephadex G-50 in 20 mM Tris-HCl pH 7.4. The excluded peak of radioactive RNA was recovered by ethanol precipitation. The specific activity of this RNA was approximately 3 x 10^6 cpm/µg RNA.

In vitro labelling of DNA

Intact HSV-1 DNA was ^{32}P-labelled *in vitro* with (α-^{32}P) deoxyribonucleoside triphosphates (300 Ci/mmol) by 'nick-translation' (11).

Preparation of cDNA's

Incubation mixes contained 4 µg HSV-1 IE polyadenylated RNA, 0.04 mM (α^{32}P)-dCTP (25 Ci/mmol), 0.4 mM each of dATP, dGTP,and dTTP, 5 µM primer oligo$(dT)_{10}$, 50 mM Tris-HCl pH 8.3, 140 mM KCl, 7 mM $MgCl_2$, 10 mM DTT, 160 u/ml AMV reverse transcriptase (kind gift of Dr J.W. Beard) and 2 µ/ml rat liver RNase inhibitor (total volume 50 µl). Mixtures were incubated at 42°C for 90 min then reactions were terminated by adding EDTA to 5 mM. Following phenol extraction, the nucleic acids

were precipitated with 3 vol of ethanol and overnight storage at -20°C. Precipitates were recovered by centrifugation, washed with ethanol, dried then dissolved in 100 µl 0.3M NaOH, 1 mM EDTA and incubated at 65°C for 20 min to hydrolyse IE mRNA. Incubations were cooled, neutralised with acetic acid, and labelled cDNA was purified by gel filtration through Sephadex G-50. Fractions comprising the initial peak of radioactivity eluted were pooled for use in hybridisation experiments. The specific activity of the cDNA was from 2-6 x 10^7 cpm/µg DNA.

Partial degradation of RNA

HSV-1 IE polyadenylated RNA was dissolved in water at a concentration of 0.4 mg/ml. 10 µl of the dissolved RNA was mixed with 10 µl 0.1 M Na_2CO_3 and incubated at 50°C for 20 min. Reactions were terminated by addition of Tris-HCl pH 7.5 to 0.1 M, and the RNA recovered by ethanol precipitation. Partially degraded RNA was used to prepare cDNA as outlined above.

Hybridisation procedures

Hybridisation of (^{32}P)-cDNA to total HSV-1 DNA bound to nitrocellulose filters was performed as outlined previously (12). Hybridisation of ^{32}P-labelled cDNA and of ^{32}P-labelled RNA to HSV-1 DNA fragments immobilized on nitrocellulose membranes by the Southern (13) blot technique was as described (1). Hybridisation to DNA fragments was detected by fluorography using Kodak X-Omat H film and Du Pont Cronex Lightning Plus intensifying screens. Films were exposed at -70°C.

RESULTS

HSV-1 genome structure and the location of IE mRNA's

Interpretation of the cDNA mapping data requires a knowledge of the HSV-1 genome structure.

As first described by Sheldrick and Berthelot (14), the genome consists of two unique DNA regions (U_L and U_S) each flanked by inverted repeat regions (TR_L/IR_L, TR_S/IR_S) and joined at IR_L-IR_S (Fig. 1A). Four genome arrangements, resulting from the possible combinations of inversions of the two unique regions, are present in DNA preparations in

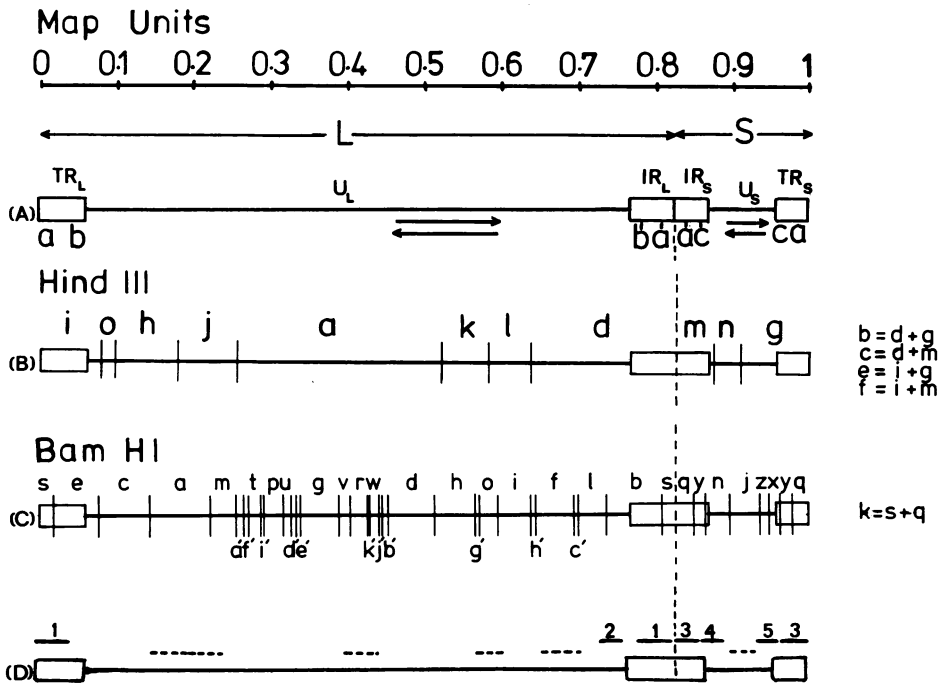


Figure 1 A Molecular organisation of the HSV-1 genome. Two unique DNA regions (U_L and U_S) are each flanked by inverted repeat regions (TR_L/IR_L, TR_S/IR_S) and joined at IR_L-IR_S. Inversions of U_L and U_S are indicated by the directions of arrows. The sequence arrangement for a single strand also is indicated, and here the non-inverted terminal redundancy is designated a. B and C Physical maps. Maps are shown for HSV-1 DNA fragments generated by restriction endonucleases HindIII and BamHI respectively. The maps are shown for one orientation of U_L and U_S. D Genome regions represented by IE mRNA's. Abundant mRNA's are indicated by numbered solid lines and minor mRNA's are shown as dotted lines

approximately equal amounts (15, 16). A consequence of this structure is that a number of fragments produced by digestion with a restriction endonuclease may contain sequences in common (17). Physical maps for the DNA fragments generated by digestion with the enzymes used in these analyses have been obtained (17, 7, A.J. Davison and N.M. Wilkie, personal communication). For the Bam HI and Hind III enzymes, these complete maps are shown for one orientation of both unique DNA regions (Fig. 1B and 1C).

Information on the genome locations of the IE mRNA's also

is required to interpret the cDNA mapping data. The five most abundant IE mRNA's have been mapped after fractionation on agarose gels containing CH_3HgOH (7) and these are represented in Fig. 1D by the numbered solid lines. IE mRNA's-1 and -3 are shown in two genome locations as these mRNA's map entirely within the repetitive DNA regions TR_L/IR_L and TR_S/IR_S respectively. The sizes and genome map locations of the numbered IE mRNA's are shown in Table 1. Genome regions represented by minor IE mRNA's which were not identified following gel electrophoresis (7) are indicated in Fig. 1D by dotted lines.

Size Distribution of IE cDNA's

Intact total IE mRNA was used as a template to synthesise ^{32}P-labelled cDNA and the distribution of acid precipitable radioactivity and of material that hybridized to HSV-1 DNA was determined after sedimentation in alkaline sucrose gradients (Fig. 2). The sedimentation positions, on a parallel gradient, of the two major vesicular stomatitis virus cDNA's (1330 and 800 nucleotides) prepared from mRNA's under similar reaction conditions (18) also are shown in Fig. 2. The bulk of IE cDNA was less than 100 nucleotides in length and no material longer than 500-600 nucleotides was detected. The reasons for the

TABLE 1

IE mRNA Species	Size (KB)	Map Locations
1	3.0	0.000 - 0.038 (TR_L) 0.792 - 0.830 (IR_L)
2	2.0	0.741 - 0.754 (U_L)
3	4.7	0.830 - 0.866 (IR_S) 0.964 - 1.000 (TR_S)
4	2.0	0.865 - 0.898 (IR_S/U_S)
5	2.0	0.938 - 0.964 (TR_S/U_S)

Table 1 Sizes and genome map locations of the major IE mRNA's -1, -2, -3, -4 and -5.

small size of the IE cDNA, prepared under these reaction conditions is unclear. Under similar conditions full length vesicular stomatitis cDNA's were obtained (18), and cDNA prepared using polyadenylated BHK RNA as a template had an appreciably larger size distribution.

Sequences present in IE cDNA's

IE cDNA preparations were hybridised to blot strips containing the HSV-1 DNA fragments generated by digestions with the Bam HI, Hind III, Xho I and Pvu II restriction endonucleases and hybridisation was visualised by fluorography. These fluorographs are shown in Figs. 3, 4 and 5 respectively and are compared to the total fragment patterns (revealed by hybridisation of ^{32}P-labelled HSV-1 DNA to the blot strips) and the hybridisation patterns of total polyadenylated cytoplasmic IE RNA's. Partial physical maps of the relevant genome regions for the DNA fragments generated by Bam HI, Xho I, Pvu II and Hind III are shown in Fig. 6.

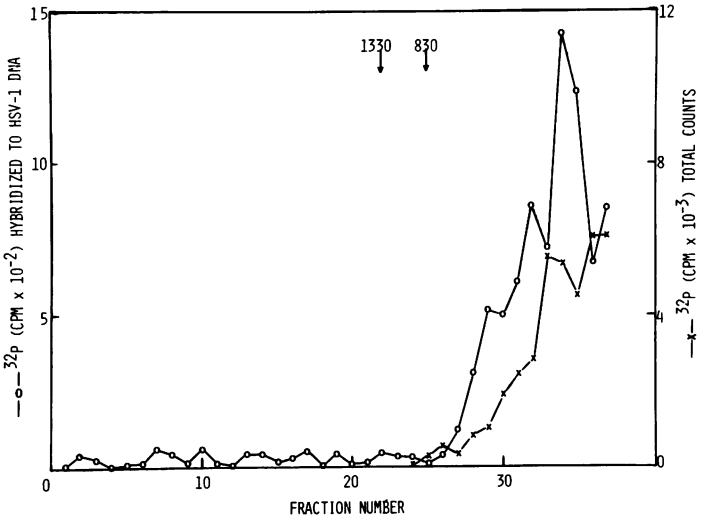
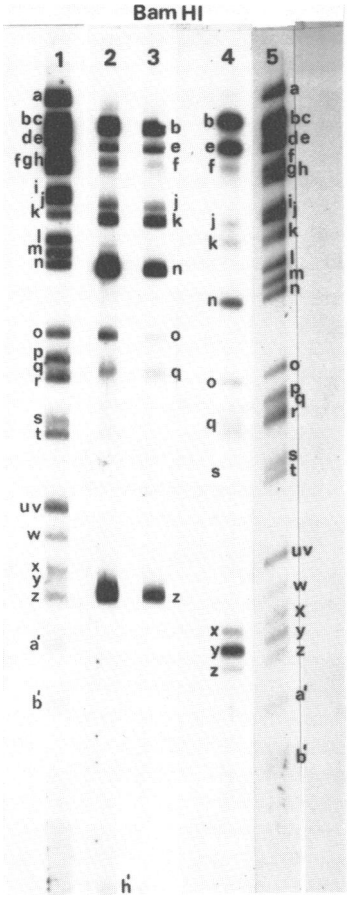


Figure 2 Size distribution of IE cDNA's Preparations were sedimented in 5-20% linear sucrose gradients in 0.75 M NaCl, 0.25 M NaOH, 0.01 M EDTA using an SW41 rotor at 40,000 rpm for 20 hr at 4°C. The direction of sedimentation is from right to left.



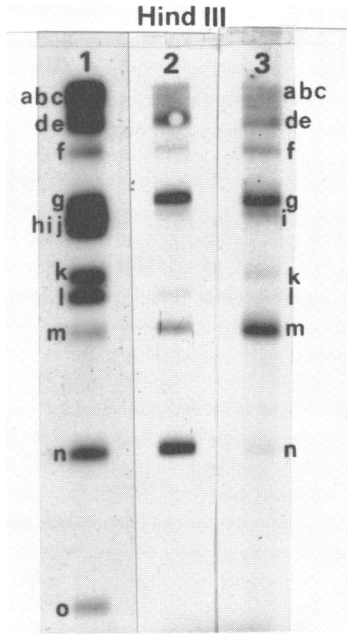


Figure 4 Fluorographs of IE cDNA's hybridised to the Hind III fragments of HSV-1 DNA Hybridisation patterns shown are 1. Nick-translated total HSV-1 DNA. 2. IE cDNA prepared using intact IE mRNA. 3. In vivo labelled total IE mRNA.

Figure 3 Fluorographs of IE cDNA's hybridised to the Bam HI fragments of HSV-1 DNA Hybridisation patterns shown are: 1. Nick-translated total HSV-1 DNA. 2. IE cDNA prepared using intact IE mRNA. 3. IE cDNA prepared using partially degraded IE mRNA. 4. In vivo labelled total IE mRNA. 5. Nick-translated total HSV-1 DNA, the control strip for hybridisation of in vivo labelled RNA.

IE cDNA did not hybridise to the whole of each mRNA region which agreed with the short size of this material as determined on alkaline sucrose gradients. This indicated that cDNA synthesis using oligo$(dT)_{10}$ as primer was initiated, as expected, on poly A. The cDNA should contain sequences complementary to the 3'-terminus of each mRNA, hence allowing orientation of these mRNA's on the genome.

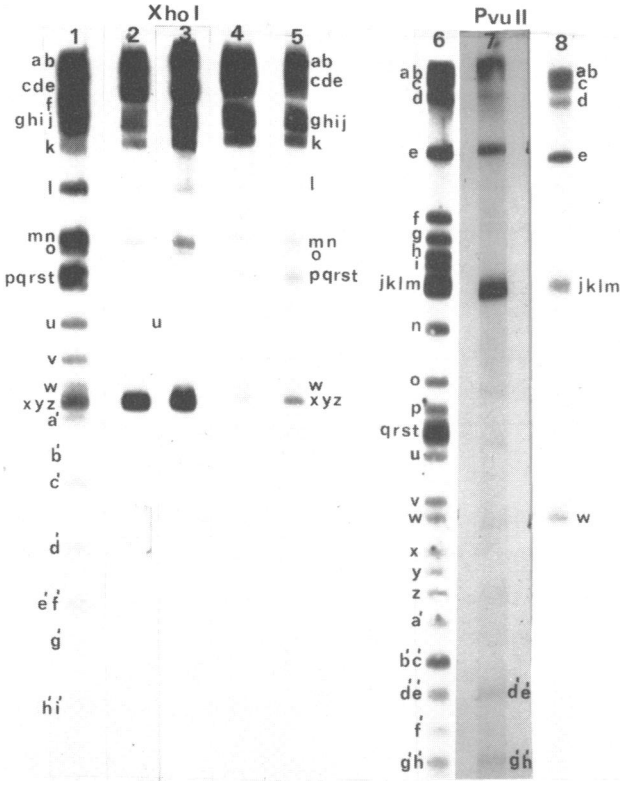


Figure 5 Fluorographs of IE cDNA's hybridised to the Xho I and Pvu II fragments of HSV-1 DNA Hybridisation patterns shown are: 1. Nick-translated total HSV-1 DNA. 2. IE cDNA prepared using intact IE mRNA. 3. IE cDNA prepared using partially degraded IE mRNA. 4. In vivo labelled total IE mRNA. 5. In vitro labelled total IE mRNA. 6. Nick-translated total HSV-1 DNA. 7. IE cDNA prepared using intact IE mRNA. 8. In vivo labelled total IE mRNA.

A formal (but unlikely) possibility remained that the reverse transcriptase was initiating not only from the 3'-terminal poly A of IE mRNA's but also from one or more internal sites, a situation which would weaken the interpretation of our results. Hence, total IE mRNA was subjected to partial alkaline degradation to a mean size of approximately 100 nucleotides and the polyadenylated fraction was re-selected on oligo dT cellulose. This polyadenylated RNA fraction was used to synthesis cDNA with the oligo$(dT)_{10}$ primer. The hybridisation patterns of this cDNA to the Bam HI (Fig. 3,

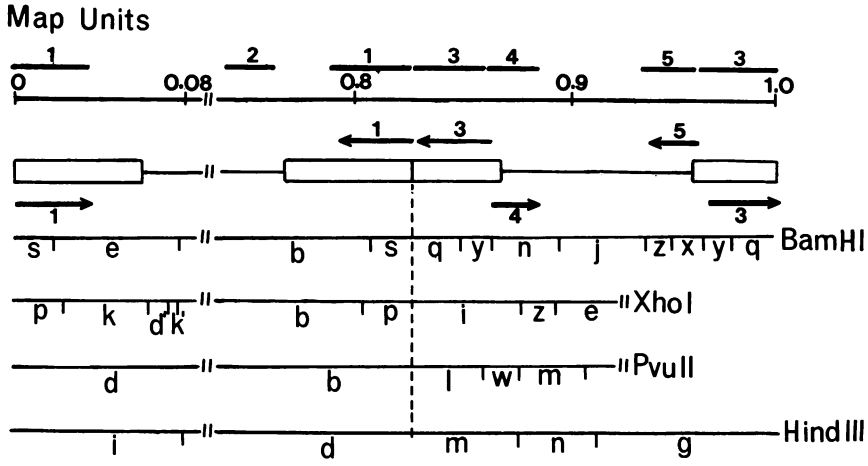


Figure 6 Genome map locations and directions of transcription These are shown for IE mRNA's -1, -3, -4 and -5, together with partial physical maps for the Bam HI, Xho I, Pvu II and Hind III fragments of HSV-1 DNA.

track 3) and Xho I fragments (Fig. 5, track 3) were very similar to those obtained with cDNA prepared using intact IE mRNA (Fig. 3, track 2; Fig. 5, track 2).

Hence, all these results are consistent with the cDNA representing the 3'-termini of IE mRNA's.

3'-terminus of IE mRNA - 1 There was abundant hybridisation of cDNA to Bam HI e but no detectable hybridisation to Bam HI s (Fig. 3, tracks 2 and 3). Similarly, there was hybridisation to Xho I k but virtually none to the Xho I pqrst band (Fig. 5, tracks 2 and 3). These data indicate that the direction of transcription must be rightwards on the TR_L template as indicated in Fig. 6. As a consequence of the HSV-1 genome structure, IE mRNA-1 synthesised using the IR_L template would be made from the complementary (antiparallel) DNA strand to that used in TR_L, and hence must be in a leftwards direction (Fig. 6).

3'-terminus of IE mRNA-2 The physical maps currently available for the HSV-1 DNA fragments which map in this region are not detailed enough to allow orientation of mRNA-2 with this technique.

3'-terminus of IE mRNA-3 The cDNA hybridised to Bam HI q but not to Bam HI y (Fig. 3, track 2 and particularly track 3). The direction of transcription therefore must be leftwards from the IR_S template and rightwards from the opposite strand of the TR_S template (Fig. 6). IE mRNA's-1 and -3 are synthesised in tandem from the IR_S/IR_L regions on the same DNA strand.
3'-terminus of IE mRNA-4 Hybridisation of cDNA was to Bam HI n and not to Bam HI y (Fig. 3, track 2 and particularly track 3). Similarly, there was hybridisation to the Pvu II jklm band but virtually none to Pvu II w (Fig. 5, track 7). There was abundant hybridisation to the Xho I xyz band (Fig. 5, tracks 2 and 3) of which only Xho I z is represented by the major IE mRNA's, and also to Hind III n (Fig. 4, track 2). Hence, mRNA-4 must be transcribed rightwards from IR_S into U_S from the complementary DNA strand in IR_S that serves in the anti-parallel direction as a template for IE mRNA-3.
3'-terminus of IE mRNA -5 Hybridisation of the cDNA was to Bam HI z and not to Bam HI x (Fig. 3, tracks 2 and 3). The direction of transcription is therefore leftwards from TR_S into U_S and this RNA is transcribed from the complementary DNA strand that provides the template for IE mRNA-4.
3'-termini of minor IE mRNA's In addition to locating the 3' termini of the major IE mRNA's, the 3' termini of several minor IE mRNA's can be identified. The IE mRNA located at 0.574-0.599 map units (Fig. 1D) spans the junction between Hind III k and Hind III l. This IE mRNA hybridises to both Hind III k and Hind III l (Fig. 4, track 3) but cDNA hybridises solely to Hind III l (Fig. 4, track 2) which indicates that the direction of transcription is rightwards.

The high specific activity cDNA probe allows location of 3' termini of minor IE mRNA's, even when the extent of the RNA map locations are not known. For example 3'-termini appear to be located in Bam HI f, Bam HI h' (Fig. 3, track 2), in Xho I l, Xho I u and Xho I o (Fig. 5, track 3).

These minor IE mRNA's, found after cycloheximide treatment, are not detected when cells are infected at non-permissive temperature with the temperature-sensitive mutant ts K which accumulates only the major IE mRNA's (4). This suggests that

these minor mRNA's may not be IE mRNA's but represent early transcripts which result from residual protein synthesis in the presence of cycloheximide.

DISCUSSION

IE mRNA-1 maps within TR_L/IR_L and codes for Vmw 110 while IE mRNA-3 maps within TR_S/IR_S and codes for Vmw 175 (6, 7). These locations raised the question whether these RNA's are transcribed from one or from both repetitive DNA templates. Information relevant to this point came from analyses of HSV-1/HSV-2 intertypic recombinants.

Recombinant B x 1(28) is heterotypic for TR_L/IR_L sequences and the DNA is fixed in one orientation of U_L. This recombinant synthesised the HSV-2 Vmw 118 polypeptide as well as the HSV-1 Vmw 110 polypeptide (6). Here, both TR_L and IR_L are expressed in one orientation of U_L. If the genome is transcribed as linear DNA, this implies that transcription of the two Vmw 110 gene copies is separately controlled from two discrete promoters which may be sequence identical.

Transcription from either TR_L or IR_L is sufficient for productive infection of tissue culture cells - sub-clones have been isolated from B x 1(28) with HSV-2 sequences in TR_L deleted and these failed to make Vmw 118. Similarly, other clones with HSV-1 sequences deleted from IR_L did not make Vmw 110 (19). However, it is significant that all field isolates so far characterised are diploid for these sequences.

The 5'-termini of IE mRNA's -1, -3, -4 and -5 all appear to map in the repetitive DNA regions, and inversion of unique DNA regions will not affect the relative locations of promoters which map in repetitions. It is possible that each of these mRNA's is transcribed from a unique promoter. In principle, these four RNA's also can be transcribed from a linear template with a single orientation of U_S and using two sequence identical but locationally distinct promoters in TR_S and IR_S (see Fig. 6). This would require two large primary transcripts and necessitate complete symmetrical transcription of U_S. These RNA's can be synthesised using a single promoter located either in TR_S or in IR_S which requires both orientations of

the U_S regions to generate the two primary transcripts.

Neither of these linear genome schemes allows for transcription of TR_L which requires a separate promoter. However, if the DNA was transcribed as a full length circular molecule, 'TR_L' sequences could be controlled by an 'IR_S' promoter while 'IR_L' sequences would be transcribed using the promoter sequence in 'TR_S' (Fig. 7).

Full length circular DNA molecules, formed from linear input DNA have been reported from cells infected with pseudorabies virus (a herpesvirus) in the presence of cycloheximide (20).

The HSV-1 physical maps currently available have not allowed the orientation of IE mRNA-2 to be determined by the method used. Synthesis of IE mRNA-2 could readily be achieved using the model outlined in Fig. 7 if it was transcribed in tandem with IE mRNA's -1 and -3.

In Fig. 7 the putative promoter region is placed at the locations where the 5'-termini of IE mRNA's -4 and -3, and IE

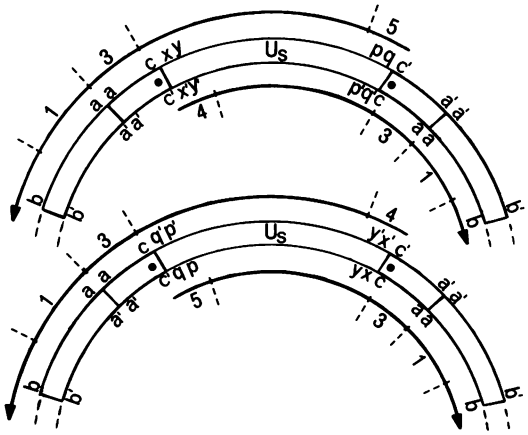


Figure 7 Model for the synthesis of IE mRNA's A circular DNA template with TR_L-TR_S joined is used to synthesise IE mRNA's -1, -3, -4 and -5 using a promoter region (●) located in c. Both inversions of U_S are shown, and the IE mRNA's are cleaved out of large primary transcripts which span U_S. An alternative model is similar but proposes bidirectional transcription from the promoter region. This obviates the need for primary transcripts which span US.

mRNA's -5 and -3 appear to map. IE mRNA's -4 and -5 overlap the junctions between U_S and TR_S/IR_S and as we have shown that their 3'-termini map in U_S, this implies that the 5'-termini map within TR_S/IR_S. It is reasonable to propose that these two transcripts are synthesised from locationally distinct promoters of identical sequence. The 5'-termini of these RNA's appear to map in Bam HI y, a DNA fragment which has an unusual structure as rapid snap-back is observed following its denaturation (J.B. Clements and N.M. Wilkie, unpublished observations).

A controlling region located in Bam HI y could function to promote bidirectional transcription analagous to that observed at the region which controls synthesis of phage λ repressor (21), and also at the E.coli ara regulatory region (22). Bi-directional transcription would obviate the requirement for the large primary transcript spanning U_S (Fig. 7) which is required to generate IE mRNA's -1 and -3. It is pertinent that a model proposed for transcription of D. melanogaster histone genes proposes two bidirectional promoters located in DNA regions which exhibit snap-back (23). Similarities in trans-criptional control between the HSV IE genes and the D. melanogaster histone genes presumably reflect a tight evolutionary constraint on the mechanisms which regulate trans-cription of gene sets.

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