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Functional analysis of a herpes simplex virus type 1 promoter: identification of far-upstream regulatory sequences

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ABSTRACT

We have performed a functional analysis of DNA sequences upstream from the gene for IE mRNA3 of herpes simplex virus type 1. Nucleotide sequences involved in initiation and positive regulation of transcription have been defined by construction of specific deletions *in vitro*. Transcription was assayed *in vivo* by microinjection into *Xenopus* oocytes, or by introduction of plasmid DNA into tissue culture cells and measurement of transient expression. Three functional promoter elements have been defined: i) Sequences between -16 and -37 which are not essential for transcription but are required for accurate initiation. ii) Proximal promoter sequences which are sufficient for transcription initiation in the absence of upstream sequences. iii) Far-upstream promoter sequences (more than 108bp upstream) which increase transcription in oocytes, and contain positive regulatory sequences (-174 to -331) which respond strongly to a factor in the virus inoculum.

INTRODUCTION

The transcriptional programme of herpes simplex virus type 1 (HSV-1) is temporally regulated and may be divided into three phases, immediate-early (IE), early and late (1, 2, 3). Immediate-early transcription takes place directly after virus infection, and, in contrast to early and late transcription, has no requirement for prior *de novo* viral protein synthesis. When cells are infected with HSV-1 in the presence of protein synthesis inhibitors (1, 4), or with certain temperature sensitive mutants at the nonpermissive temperature (5, 6), large amounts of IE mRNAs accumulate.

Transcription of HSV-1 DNA throughout the lytic cycle is mediated by an α-amanitin sensitive cellular RNA polymerase (7), and in view of the observation that deproteinised HSV-1 DNA is infectious (8), it is clear that IE transcription can be

directed by unmodified cellular RNA polymerase II. Transcription of at least four of the five HSV-1 IE genes (IE mRNAs 2-5, Fig. 1A), however, is positively regulated in the infected cell by a component of the virus inoculum, presumably in the virus particle (9, 10, MGC and CMP, unpublished data), and it appears that although RNA polymerase II can recognise IE promoter sequences, the efficiency of transcription is greatly increased by the presence of regulatory factors. Recently, the precise location of the 5' terminus of IE mRNA3, a four kilobase RNA which encodes the IE polypeptide Vmw 175, has been described (11, 12). Furthermore, the DNA sequence which encompasses the 5' terminus and upstream region of IE mRNA3 has been determined (13, 14). We have utilised this information to perform a functional analysis of the IE mRNA3 promoter region in order to locate and characterise the sequences responsible for directing transcription in the absence and presence of viral regulatory factors. Specific deletions have been introduced into the promoter region, and their effect upon initiation and regulation of transcription has been determined by in vivo assays.

Transcription in the absence of regulation has been assayed by microinjection of plasmid DNAs into Xenopus laevis oocytes. This approach has been successfully used by others to characterise transcription control sequences for a number of eukaryotic genes(15, 37). Experiments designed to locate sequences which respond to activation by the component of the virus inoculum have used a series of plasmid constructions in which altered IE mRNA3 promoters are linked to the HSV-1 thymidine kinase (TK) structural gene sequences (IE-TK genes). These plasmids have been introduced into tissue culture cells, and their response to positive regulation determined by measurement of the stimulation of TK synthesis after superinfection with HSV-1 mutants.

MATERIALS AND METHODS

Plasmid DNAs: The recombinant plasmids used in this study were pTK1 (pGX153) and pN1 (pGX33) which consist of the BamHIP and BamHIN fragments of HSV-1 DNA (Glasgow strain 17) cloned into the plasmid vector pAT153 (16). Plasmid DNA for microinjection and transfection procedures was purified by sucrose density gradient centrifugation.

Cells and Virus: For the production of IE infected cell RNA, BHK21 (Cl3) cell monolayers were infected with HSV-1 (Glasgow strain 17) at a multiplicity of 50 pfu/cell. The monolayers were pretreated and maintained in medium containing cycloheximide as previously described (1). Cytoplasmic RNA was extracted as described previously (17).

Xenopus Oocyte Microinjection: Female X.laevis were purchased from Xenopus Ltd, Redhill, England. Stage 5 oocytes were microinjected with 20 nl of plasmid DNA solution (5 µg/ml) into the nucleus as described by Gurdon (18). Groups of 25 oocytes were incubated in modified Barth medium (18) at 22°C for 40h. They were then manually disrupted and either prepared for enzyme assay, or extraction of total oocyte RNA (19).

Nuclease S1 Analysis of IE mRNA3: Previously described nuclease digestion procedures were employed (20, 21). A single stranded 5'-terminally labelled viral DNA probe (BamHIN, EcoRIb; Fig. 1a) from the IE mRNA3 coding DNA strand was annealed with sample RNA in 20µl of 90% (v/v) formamide, 0.4M NaCl, 40mM PIPES, pH 6.8, 1mM EDTA for 16h at 42°C after denaturation at 90°C for 3 min. Hybridisation reactions were rapidly quenched on ice and incubated with 4000 units of nuclease S1 at 37°C for 1h in 200 µl of 0.2M NaCl, 30mM NaOAc, pH 4.5, 1mM $ZnSO_4$, digested material was extracted with phenol/chloroform and precipitated with ethanol. Denatured hybrids were electrophoresed on thin (0.35 mm) 10% polyacrylamide-urea gels (22). Terminally labelled HpaII restriction enzyme fragments of pBR322 were co-electrophresed as size standards.

Thymidine Kinase Assays: HSV-TK was assayed by following the phosphorylation of [methyl-^{3}H] thymidine, as described previously (23, 24). TTP (100µM) was added to suppress the cellular or oocyte thymidine kinase activities (19, 24). TK determinations were carried out on an amount of oocyte extract equivalent to 1 oocyte.

Transformation of BHKTK$^-$ Cells: Monolayers of $2x10^6$ BHKTK$^-$ cells, (a gift of C. Macdonald, Dept. of Genetics, University of Glasgow) were transfected with BamHI cleaved plasmid DNA in the presence of 4µg BHKTK$^-$ cell carrier DNA using the calcium phosphate technique followed by DMSO boost (24) at 4h post-transfection and addition of HAT medium (25) 20h post-transfection. Cells were grown in HAT medium for approximately 12 days after which TK$^+$ colonies were either a) stained and counted or b) grown out and passaged in HAT medium.

Transient Expression Transfection Assays: Monolayers of 10^6 BHK cells were transfected with 0.1 to 1.0µg of plasmid DNA using the modified calcium phosphate/DMSO technique (25). The total DNA added to cells was adjusted to 3µg by addition of calf thymus DNA. One hour after the DMSO boost, U.V. light-inactivated HSV-1 mutant tsK was added to cells at a concentration equivalent to 3 pfu per cell of non-inactivated virus. Cell cultures were incubated at 38.5°C for 16h and TK activity of extracts was then assayed.

Plasmid Constructions: All IE mRNA3 promoter deletion mutants (Fig. 1B, Fig. 3) were derived from pN1 by established DNA manipulation procedures. The mutants, pN3Δ 58, pN3Δ 61, pN1Δ47/67 were generated by deletion with exonuclease III, from the unique EcoR1 site in BamHlN, as described by Sakonju et al.

(26). EcoR1 linkers were inserted at the deletion endpoints. Sequence analysis of deletion end points was carried out by the method of Maxam and Gilbert(22). Construction of IE mRNA3 promoter-TK gene chimaeras (IE-TK genes) utilised the existence of a unique BglII cleavage site at position +54 in the untranslated 5'leader region of TK mRNA (Fig. 3a), and a BamHI site at position +27 in IE mRNA3 coding sequences. BamHI-BglII or EcoRI-BglII fragments containing TK structural gene sequences were therefore inserted downstream from the IE mRNA3 promoter (Fig. 3b, c, d). Alternative constructions (Fig. 3e, f) employed an EcoRI fragment of HSV-1 DNA containing TK gene sequences and 78 bp of upstream sequence which do not constitute a normally functional promoter *in vivo* (15).

RESULTS

Recognition of the IE mRNA3 Promoter by Xenopus Oocyte RNA Polymerase II A cloned 4.5 kb BamHI fragment of HSV-1 DNA (pN1), which contains the 5'-terminus and all putative transcriptional control signals of IE mRNA3 (9, 11, 12) has been employed in these studies (Fig. 1a). *Xenopus* oocytes were microinjected with plasmid pN1 DNA, and stable RNA transcripts were subjected to nuclease S1 analysis with 5' end labelled BamHI*N*,EcoRI*b* coding strand probe DNA (Fig. 1a). Nuclease S1-resistant hybrids were fractionated on a 0.35 mm thick polyacrylamide-urea gel (Fig. 2a). Transcripts produced in oocytes (track 2) have identical 5'-termini to IE mRNA3 synthesised in infected cells (track 1) and protect DNA fragments of 26-29bases (b), corresponding to preferred sites of initiation within the left hand end of BamHI*N* sequences. It is therefore clear that this HSV-1 IE promoter accurately directs transcriptional initiation in oocytes.

Analysis of Transcription Initiation Signals in Microinjected Xenopus Oocytes A series of specific deletions were generated in the upstream sequences of the gene for IE mRNA3 (Fig. 1b), and their ability to initiate transcription was determined by nuclease S1 analysis of RNA from microinjected oocytes (Fig. 2b). Plasmids retaining 161base pairs (bp)(pN4), and 108bp (pN3) of sequence upstream from the gene (tracks 4, 5) directed transcription at high levels in oocytes, whereas no detectable transcripts were directed by pN3Δ 58, pN3Δ 61 and pN3Δ 90 which retain 50, 47 and 18bp of upstream sequence respectively (tracks 3, 2, 1). This result indicates the

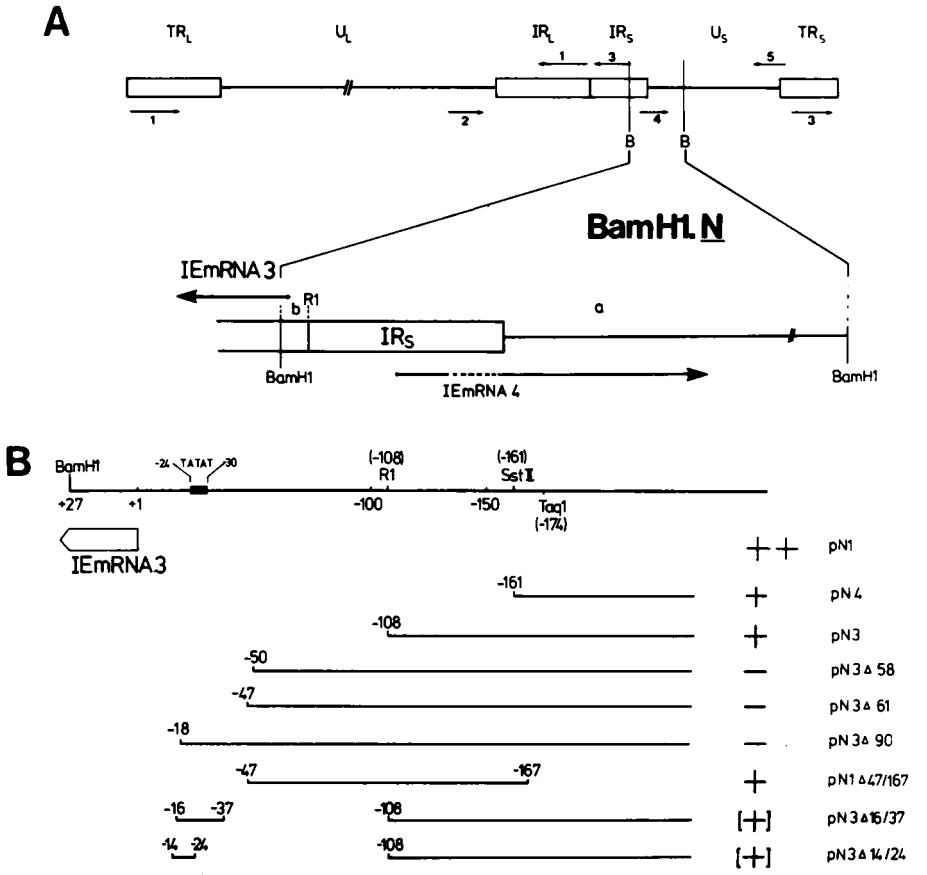


Figure 1.(A) Locations and directions of synthesis of the IE mRNAs(1-5) on the HSV-1 Genome: The BamHIN fragment of the genome is expanded, BamHI(B) sites,and the unique EcoRI(RI)site within BamHIN sequences are indicated. IEmRNA3 is transcribed in a leftward direction from a position approximately 30bp within the fragment (11,12).(B)Structure of Deletion Mutants in the IE mRNA3 Promoter Region. IEmRNA3 transcription is depicted in a leftward direction from nucleotide position +1 which represents one of the 3 predominant initiation sites on the DNA sequence. The sequences deleted in each construction are indicated and the end point of the deletions noted above the line. Transcriptional ability of each promoter is indicated by the motifs "+" (accurate initiation of transcripts),"[+]" (non-specific initiation of transcription),"-" (no detectable transcription).

existence of a functional promoter element between nucleotide positions -50 and -108 of the gene, and suggests that sequences within 50 bp from the gene, which include the TATA homology (-25

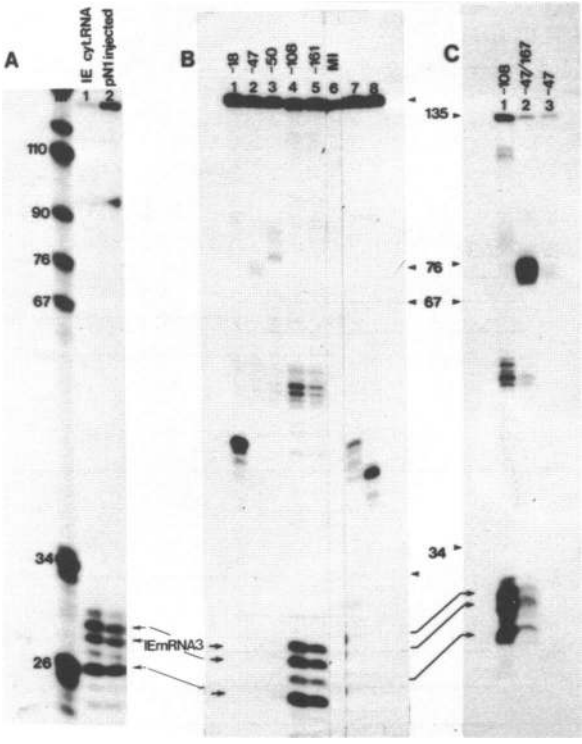


Figure 2.Nuclease S1 Analysis of IEmRNA3-specific transcripts in Xenopus Oocytes.The BamHIN,EcoRIb fragment was 5' terminally labelled and strand separated,the IEmRNA3 coding strand was hybridised with 10µg samples of microinjected or uninjected total oocyte RNA, or 5µg IE HSV-1-infected cell RNA. Nuclease S1 resistant material was denatured and electrophoresed on a 10% polyacrylamide-urea, 0.35mmx25cmx45cm gel. Panel A: HSV-1 infected cell cytoplasmic RNA (track 1) and pN1-injected oocyte RNA(track 2) were hybridised with probe DNA. The track "M" contains terminally labelled marker fragments. Panel B: Analysis of oocyte transcripts directed by IEmRNA3 promoter deletion mutants; pN3Δ90(track 1),pN3Δ61(track 2),pN3Δ58(track 3), pN3(track 4),pN4(track 5),mock injected(track 6) pN3Δ16/37(track 7),pN3Δ14/24(track 8). Panel C: Analysis of oocyte transcripts directed by; pN3(track 1),pN1Δ47/167(track 2), pN3Δ61(track 3). Deletion end points are noted above tracks.

to -29), are unable to direct significant levels of transcription in the absence of adjacent upstream sequences. To investigate further the role of the TATA homology in the IE mRNA promoter, specific deletions were introduced into plasmid pN3.

One of these, pN3Δ16/37, has a 22bp deletion of sequences -16 to -37, which therefore removes the TATA homology, and another, pN3Δ14/24, has lost sequences -14 to -24, which lie immediately downstream from the TATA homology and alter its spacing with respect to the regular RNA capping sites (Fig. 1b, 5). Neither pN3Δ16/37 nor pN3Δ14/24 directed correctly initiated transcripts from the IE mRNA3 promoter in microinjected Xenopus oocytes (Fig. 2b, tracks 7 and 8). The only transcripts detected in these hybridisations map to the point of sequence divergence between the probe and the mutant template (hybrids of similar origin are observed with other deletion mutants; track 1; Fig.2c, track 2) and are caused by low levels of transcription from upstream sites in pAT153. It was also possible that transcripts were initiated downstream or at heterogeneous sites and were not detected by the probe employed. To investigate this possibility, IE mRNA3 promoter variants were linked 5' to the HSV-1 TK gene structural sequences,(Fig. 3) and transcription was assessed by measurement of DNA-directed TK synthesis in oocytes. The results are presented in Table 1, which shows that pNTK3Δ16/37TK and pTKN3Δ14/24TK both retain competent promoters but are somewhat impaired with respect to their parent plasmid pTKN3. The TATA homology is therefore neither essential nor sufficient for initiation of transcription in oocytes. Sequences including the TATA homology are clearly required for accurate initiation as in other systems (15,35,37), and displacement of the regular cap site with respect to the TATA homology also abolishes initiation at the normal position.

Role of Far-upstream Sequences in the IE mRNA3 Promoter

Comparison of TK synthesis directed by molar equivalent levels of pTKN3 and pTKN1 (the undeleted BamHIN fragment linked to TK structural gene sequences) in microinjected oocytes revealed that sequences upstream from position -108 increase TK expression two-fold and therefore have a quantitative effect upon IE mRNA3 transcription in oocytes (Table 1). Furthermore, pN1 was found to be three- to five-fold more efficient than pN3 or pN4 when transcription at the IE mRNA3 promoter was analysed by nuclease S1 analysis (results not shown). These findings suggest that sequences upstream from -161 stimulate

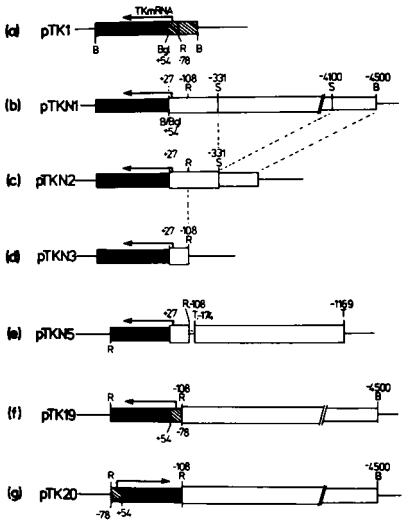


Figure 3. Schematic Representation of IE-TK Chimaeric Plasmids: Linearly permuted plasmid structures are represented (not to scale). Solid lines: pAT153 vector sequences. Open bars: HSV-1 BamHI N sequences. Solid bars and hatched bars: HSV-1 BamHI P sequences. Hatched bars are HSV-1 sequences upstream from the BglII site at position +54 TKmRNA sequences,solid bars are sequences downstream from position +54 and therefore include TK structural sequences and the first AUG initiation codon. TKmRNA transcripts are indicated by arrows and originate at position +1 in the TK promoter (a),(f),(g) or in the IEmRNA3 promoter(b),(c), (d),(e). Nucleotide positions within the TK promoter are indicated below the lines and nucleotide positions in the IEmRNA3 promoter,above the lines. Restriction enzyme cleavage sites are denoted B(BamHI), S(SmaI), Bgl(BglII), R(EcoRI) and B/Bgl(BamHI, BglII hybrid restriction site). The asterisk (*) in construction(e) denotes a 26bp insertion of pAT153 vector sequence.

transcription from the promoter. The role of these sequences was demonstrated more clearly by microinjecting plasmid pN1Δ 47/167 (Fig. 1b), which lacks the proximal promoter but retains the upstream sequences, into Xenopus oocytes. In this experiment, IE mRNA3-specific transcripts were detected (Fig. 2c, track 2) whereas the equivalent plasmid without the upstream sequences, pN3Δ61, was inactive (Fig. 2c, track 3). It should be noted that the levels of correctly initiated transcripts were lower than from pN3 (Fig. 2c, track 1) and also that many transcripts initiated upstream from position -47 map to the point of sequence divergence between pN1Δ47/167 and the probe DNA, causing a 75b hybrid in track 2. The reduced fidelity of transcription is clearly a result of the large internal deletion of promoter sequences in pN1Δ 47/67. A second functional region of the promoter can therefore be defined upstream from position -167. This sequence will promote transcription independently in oocytes, in the absence of

Table 1: DNA-directed TK Synthesis in Xenopus Oocytes

Plasmid DNA Microinjected [1]	TK Activity[2] cpm x 10^{-4} [^{3}H] phosphorylated
pTKN1	291
pTKN3	168
pTKN3 Δ 16/37	94
pTKN3 Δ 14/24	58
pRCTK[3]	5
Uninjected	4

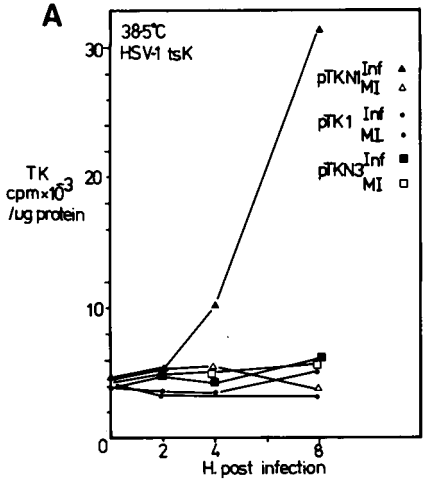
1. Batches of 25 oocytes were microinjected with 1ng/oocyte plasmid DNA, at which level TK synthesis by oocytes responds linearly to microinjected template (MGC unpublished data). Oocytes were collected at 40h, and lysates were subjected to TK determinations; each determination used an amount of lysate equivalent to 1 oocyte.
2. Activities were adjusted to represent TK synthesis by a µg pTKN1 equivalent of plasmid DNA.
3. The plasmid pRCTK consists of a promoterless TK gene, constructed by removal of sequences upstream from +54 in TKmRNA untranslated leader sequences.

functional proximal promoter sequences (-50 to -108), and also increases the efficiency of transcription directed by the proximal promoter sequence.

Positive Regulation of IE mRNA3 Transcription To examine the regulatory sequences of the IE mRNA3 promoter, it was necessary to use tissue culture cells which are susceptible to HSV infection. Plasmids with IE mRNA3 fragments linked to TK structural sequences were therefore introduced into BHK cells, and their expression examined after superinfection with HSV.

In the first series of experiments, BHKTK$^-$ cells were converted to stable TK$^+$ transformants by transfection with pTKN1, pTKN3 or pTK1 (Fig. 3a,b,d). A plasmid containing only the TK structural sequences (pRCTK, see table 1)gave no transformants, whereas pTKN3 gave a level close to that of pTK1. Plasmid pTKN1, however, was approximately 100x more efficient than pTKN3 or pTK1 in generating TK$^+$ colonies. Although many factors must affect the ability to generate a stable TK$^+$ phenotype, promoter efficiency presumably plays some role in this process (15, 27, 28). These observations therefore indicate the existence of promoter sequences upstream from -108 and support the data from microinjection experiments.The cell lines

were superinfected at the nonpermissive temperature with U.V. inactivated HSV-1 tsK. Inactivation by U.V. irradiation prevents synthesis of viral products, but does not affect the regulatory component of the virus inoculum (M. Campbell and C. Preston, unpublished data). Transcription of the resident TK gene can therefore be monitored in the absence of TK expression from the superinfecting virus. Resident TK gene expression was strongly activated in superinfected pTKN1-transformants (Fig. 4a), as shown previously (9), but no stimulation of TK expression was detected in pTKN3-transformants, or cells transformed with the "early" TK gene (pTK1) which requires expression of viral functions for positive regulation (31).In order to confirm that the increased TK expression observed in pTKN1-transformed cells was a result of increased levels of TKmRNA, pTKN1, pTKN3 and BHKTK$^-$ cell lines were superinfected with HSV-2 ts9 (32) and maintained at the nonpermissive temperature for 8h followed by nuclease S1 analysis of IE mRNA3-specific transcripts. This HSV-2 temperature sensitive mutant has a very early functional defect (32) and its use avoids interference of superinfecting virus IE transcripts in the hybridisation analysis. Cytoplasmic RNA was extracted from HSV-2 ts9-infected and mock-infected cells and subjected to nuclease S1 analysis with the BamHI<u>N</u> EcoRI<u>b</u> coding strand probe labelled at its 5' end (Figure 4b). Transcripts initiated at the IE mRNA3 promoter were highly amplified in superinfected pTKN1-transformed cells (track 1) compared to mock-infected cells (track 4) and a small, 2-fold increase in transcription was detected in superinfected pTKN3-transformed cells (tracks 2,5). The 40b hybrid (track 2) does not correspond to an expected transcripton initiation site and at present remains unexplained, however the prominent 57-58b hybrid (track 5) is routinely observed in hybridisations with this probe (see also Fig. 2b,c), and is an artifact caused by sequence specific cleavage of DNA/DNA hybrids by nuclease S1 at an A+T rich sequence (the TATA homology). Such hybrids arise because low levels of the complementary strand contaminate the coding strand probe DNA. These experiments therefore show that sequences upstream from -108 in the promoter are responsible for



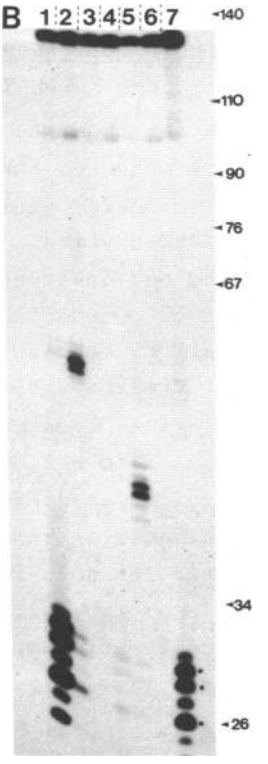


Figure 4. Positive Regulation of the IE mRNA3 Promoter in TK-transformed Cells: Stably TK-transformed cell lines were established with the HSV-1 TK gene (pTK1) or with pTKN1 and pTKN3,chimaeric TK genes.(A) TK synthesis in cell lines superinfected at 5pfu/cell with U.V. inactivated HSV-1 tsK at 38.5°C.(B) Nuclease S1 analysis of IEmRNA3 transcripts in TK-transformed cell lines.
Cytoplasmic RNA was extracted from cell lines 16 h postinfection with 5pfu/cell HSV-2 ts9 at the non-permissive temperature and 10µg was subjected to nuclease S1 analysis with BamHI N,EcoRI b coding strand probe DNA. Nuclease S1 resistant hybrids were denatured and electrophoresed on a 10% polyacrylamide-urea gel (0.35mmx25cmx45cm). Hybridisations were pTKN1-transformed cell RNA(track 1,4), pTKN3-transformed cell RNA (track 2,5), and BHKTK-cell RNA(track 3,6).Superinfected cell RNA is shown in track 1,2,3 and mock-infected cell RNA in track 4,5,6. Hybridisation with 5µg of HSV-1 IE infected cell cytoplasmic RNA is shown in track 7.

activating the expression of the linked TK gene by increasing mRNA levels. Since TK mRNA is relatively stable in HSV-1-infected cells ($t^1/2$ approximately 3h, results not shown), the increase is probably a direct result of increased rates of transcriptional initiation at the IE mRNA3 promoter. We note also that although the activation is mediated overwhelmingly by upstream sequences, the short 108 bp promoter fragment (pTKN3) retains a limited response to superinfection as determined by

direct nuclease S1 analysis.

Location of Upstream Activating Sequences In order to identify the far-upstream promoter sequences responsible for activating IE mRNA3 transcription in response to superinfection, we have measured the increased expression of TK from transfected IE-TK chimaeric genes in BHK cells after superinfection by U.V. inactivated virus. This measure of transient expression is carried out under non-selective conditions, and eliminates a number of potential disadvantages inherent in the quantitative analysis of regulation of gene promoters resident in transformed cells after selection in HAT medium (see Discussion). The transient expression assay involved transfection of IE-TK plasmids into BHK cells, followed at 5h by superinfection with U.V. inactivated HSV-1 tsK at the non permissive temperature. TK synthesis in superinfected and mock superinfected cells was measured 16h post-infection.

From table 2, it can be seen that transfection of pTKN1 into cells stimulated TK activity 4-fold. Plasmid pTKN3, however, gave only a small, barely detectable increase in TK level. Therefore, as in Xenopus oocytes, sequences upstream from the proximal promoter increase transcription of the IE mRNA3 gene. When cells transfected with pTKN1 were superinfected, a 75-fold stimulation of TK activity was observed, whereas no significant increase of TK in cells transfected with pTKN3 or

Table 2: Regulation of transient expression from the IE mRNA3 promoter intransfected BHK cells:

Plasmid DNA	TK activity[1]		Stimulation
	-superinfect[n]	+superinfect[n]	(fold)
None	1.1	1.1	1[2]
pTK1	1.4	1.3	1
pTKN1	4.1	306.2	75
pTKN2	3.9	282.1	72
pTKN3	1.3	1.5	1.2
pTKN5	3.1	165.5	53
pTK19	2.1	71.6	37
pTK20	1.1	1.1	1

1. Expressed as cpm $x10^{-3}$/µg protein/µg pTKN1 equivalent.
2. A value of 1 represents no stimulation.

pTK1 was found (Table 2). These results agree with the findings using transformed cells (Fig. 4), although the stimulation of TK production is somewhat greater.

Further plasmid constructions were used to locate sequences which respond to superinfection. Plasmid pTKN2 contains only sequences up to a SmaI site at -331, plus a distal region located approximately -4100 to-4500 from the IE mRNA3 5'- terminus. This plasmid gave equivalent results to pTKN1 (with or without superinfection), showing that it retains the sequences which respond to superinfection. Another plasmid, pTKN5, contains upstream sequences to -1170, and, in addition, the region -108 to -174 is replaced by 26bp of pAT153 DNA. This plasmid is expressed and responds to superinfection almost as efficiently as pTKN1 and pTKN2. It is not clear whether the slight reduction in TK activity of pTKN5 is due to deletion of some promoter sequences in the region -108 to -174, or to alterations of the normal spacing between the proximal promoter and an upstream region. Since the only HSV-specific sequences upstream from -108 common to pTKN1, pTKN2 and pTKN5 are those between -174 (a TaqI site) and -331 (a SmaI site), we conclude that these coordinates provide limits for the regulatory sequences which respond to superinfection.

The above plasmids have alterations in far upstream regions while retaining the normal proximal promoter for IE mRNA3. To determine whether the far upstream sequences can function in the absence of the proximal promoter, two further plasmids, pTK19 and pTK20 (see Fig. 3) were analysed. These consist of the IE mRNA3 promoter linked to an EcoRI fragment of pTK1 which contains the TK structural gene and some upstream sequences, but lacks a complete promoter (15, 28, 29). When far upstream sequences were linked in their normal orientation, upstream from the mRNA 5'-terminus, (pTK19), response to superinfection was observed (Table 2), showing that regulation can occur in the absence of any other promoter elements specific to IE mRNA3. The failure of pTK20 to respond shows that the orientation with respect to the mRNA 5' terminus and TATA homology is important.

DISCUSSION

Functional analysis of the promoters of many cellular or viral genes transcribed by RNA polymerase II have revealed the existence of cis-acting transcriptional control sequences located 5' to the gene coding sequences. The results presented here described three distinct functional elements in the HSV-1 IE mRNA3 promoter and these will be discussed in the context of other eukaryotic transcriptional promoter sequences.

The TATA Homology The sequence TATATGAG found at position -22 to -29 in the promoter (Fig. 5) is analogous in position to the TATA homology (canonical sequence $\begin{matrix}T\\C\end{matrix}ATA\begin{matrix}T\\A\end{matrix}AAG$) observed in many eukaryotic promoters (33, 34). Our studies have shown that sequences -16 to -37 are not essential for promoter function in Xenopus oocytes, but appear to be important for precise initiation of transcription. In this respect our results agree with those from other systems in vivo, where the role ascribed

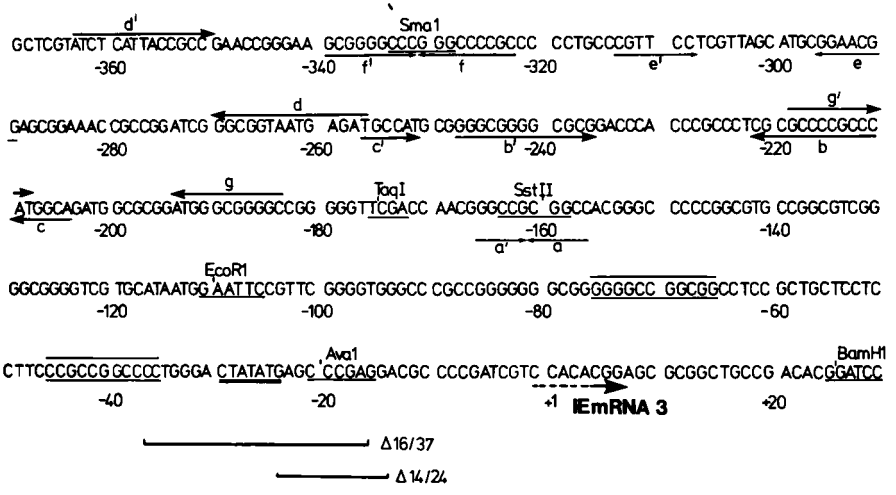


Figure 5. Nucleotide Sequence of the IE mRNA3 Promoter Region: Nucleotide positions are assigned relative to one of the IEmRNA3 major capping sites, the TATAT sequence is doubly underscored, and putative proximal promoter sequences are under and overlined. Other inverted symmetries in the upstream region are indicated, corresponding numbers of inverted symmetries are denoted a/a', b/b' etc. The sequences represented are eqivalent to nucleotide positions 1840 - 2239 on the sequence of Murchie and McGeoch(1982).

to the TATA homology is that of positioning the site of initiation approximately 30 bases downstream (15, 35-37).

Promixal Promoter Sequences Plasmid pTKN3 16/37 can direct synthesis of TK in Xenopus oocytes, showing that sequences -38 to -108 plus sequences downstream from -15 contain promoter activity. Within these regions, there exists an important element between positions -50 and -108, since deletion of these sequences abolishes transcription. We propose that this "proximal promoter" is analogous in function to the upstream promoter elements described for a number of genes (15, 36-40). It shares no obvious sequence homology with other upstream promoter elements, and the so-called CAAT homology (canonical sequence GG$^{T}_{C}$CAATCT) conserved at around position -80 in many eukaryotic promoters (34) and known to be functionally important for transcription of -globin genes in vivo (41) is not present.

The most obvious sequence feature discernible in this region is a hyphenated 11bp dyad symmetry at positions -36 to -46 and -66 to -76 (Fig. 5) separated by a 20bp tract of relatively A+T rich DNA. These sequences are similar in position to the dyad symmetries which have been implicated in the upstream promoter element of HSV-1 TK (42), and regulated upstream promoter elements of mammalian metallothionein genes (39, 43), and Drosophila heat shock genes (40). Such sequences are already strongly implicated in DNA/protein recognition in prokaryotes, and alternatively one can speculate that hairpin-loop formation might occur and induce a local unwinding of the DNA helix and thus facilitate polymerase binding at this site. Precise characterisation of the proximal promoter however awaits further analysis, and direct evidence for importance of the inverted symmetry in promoter function will rest on the production of specific mutations within these sequences.

Far-Upstream Promoter Sequences Sequences upstream from -167 have been shown both to direct transcription in oocytes in the absence of the proximal promoter sequences, and also to exert a quantitative effect upon transcriptional efficiency. The existence of such promoter sequences is strongly supported by TK- transformation assays which revealed that sequences upstream from -108 enhanced transformation efficiency of the promoter

100-fold.

Sequences located within the far-upstream region which influences promoter activity were found to mediate a regulated activation of transcription from the IE mRNA3 promoter in tissue culture cells in response to a factor in the superinfecting virus inoculum. The site of regulation was located by transient expression assay to within a 157bp stretch of upstream sequence, -174 to -331 which is also capable of activating transcription from a heterologous proximal promoter. Recently Mackem and Roizman (48) investigated TK expression directed by IE mRNA3 promoters in TK-transformed cells, and concluded that sequences upstream from -108 cumulatively mediated positive regulation. Our results using transient expression assays are in general agreement with these findings, however we show that the response to superinfection can predominantly be assigned to sequences between nucleotides -174 and -331. It is worthwhile pointing out that there are limitations to both systems, primarily because the significance of the TK levels without superinfection is unclear. On one hand, measurement of endogenous and activated TK levels in uncloned transformed cells takes no account of possible copy number differences and other factors which may influence the expression of TK genes probably resident in the cellular chromosome (27). The observation that pTKN1 gives rise to approximately 100x times more colonies per microgram than pTKN3 suggests that differences may exist in the resultant cell lines. Conversely, the transient expression assay overcomes these problems but can detect expression without superinfection only when plasmids with an efficient promoter (pTKN1, pTKN2, pTKN5 and pTKN19) are used. The stimulation of TK levels after transfection with plasmids containing weaker promoters (pTK1, pTKN3 and pTK20) is small, however, and cannot be measured accurately. Clearly both systems can meaningfully detect sequences which respond to superinfection but small quantitative differences between such sequences should be interpreted with caution.

The sequences responsible for regulated activation of the gene (-174 to -331), have an effect on short term expression which resembles, in magnitude, that of "enhancer" sequences,

which have been recognised in papovavirus genomes and retrovirus LTRs (44, 45, 46). Furthermore, the effect of sequences upstream from -108 on TK-transformation , resembles the large increase in efficiency conferred on the HSV-1 TK gene by linkage to the SV40 "enhancer" sequences (47). Further experiments will resolve whether these effects are attributable to the same or different far-upstream sequences, and whether the sequences responsible share other properties which are charecteristic of "enhancers", for instance the ability to exert an effect over relatively large distances, and independent of orientation with respect to the gene (44,45). The precise nucleotide sequences responsible for the regulated activation of the promoter remain to be determined. The most noteworthy feature of the activating region presently defined is the presence of multiple and overlapping hyphenated dyad symmetries (Fig. 5) whose complex and extensive intra-strand complementarity has considerable potential for assuming altered secondary structure. Relevant to this possibility , and to the putative role of dyad symmetry in the proximal promoter, is the observation that hyphenated dyad symmetries in negatively supercoiled plasmid DNAs are capable of forming intra-strand stem loop structures at physiological salt concentrations (48,49). It is noted however, that dyad symmetry elements are not exclusively located in this region, but are also found outwith the sequences responsible for activation.

Two possible mechanisms by which activation might occur are envisaged. The regulatory factor may interact directly with DNA, perhaps to induce conformational change in this region, rendering the promoter more transcriptionally active, or alternatively it may interact with RNA polymerase II in a sigma-factor like capacity. Activation by induction of an RNA polymerase binding/entry site on the DNA seems the most likely mechanism, and further, more refined, site directed mutagenesis experiments will identify the specific sequence element(s) contributing to these effects.

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