Volume 14 Number 9 1986 Nucleic Acids Research

DNA replication is required for abundant expression of a plasmid-borne late US11 gene of herpes simplex virus type 1

Paul A.Johnson and Roger D.Everett

MRC Virology Unit, University of Glasgow, Church Street, Glasgow G11 5JR, UK

Received 21 March 1986, Accepted 10 April 1986

ABSTRACT

During herpes simplex virus type 1 (HSV-1) infection, the appearance of true-late gene products is severely reduced under conditions of DNA synthesis inhibition. This report describes the use of a plasmid-borne promoter of a true-late HSV-1 gene (US11), linked to the rabbit β-globin gene, to study the requirement of DNA replication for late gene expression. The activity of the plasmid-borne US11 promoter in constructs containing or lacking an HSV-1 origin of replication ($ORIs$) was analysed by quantitative S1 mapping of correctly initiated hybrid transcripts. Following HSV-1 superinfection of transfected HeLa cells, the US11 promoter in ORI^+ plasmids was expressed with similar kinetics to the viral US11 promoter. US11 promoter activity was first detected at the same time as the onset of DNA template replication. Expression of US11 RNA was detectable from non-replicating ORI^- plasmids, although transcript accumulation was reduced by greater than 90%. Sequences containing the IE-5 promoter (a 3' co-terminal gene whose transcription starts 5' of US11) also played a positive role in achieving normal US11 gene expression.

INTRODUCTION

A feature of cells productively infected with herpes simplex virus type 1 (HSV-1) is the coordinate, temporal control of the viral transcriptional programme. The classification of at least three groups of HSV genes, immedediate-early (IE), early and late, is based on the kinetics of appearance and requirements for expression of their RNA and protein products (1,2,3). We are interested in the transcriptional regulation of the class of HSV genes whose products appear latest during viral infection. The gene employed here for investigation is US11), which lies in the short-unique region of the HSV genome and odes a protein with apparent mol.wt. 21,000 (5). The ics of US11 gene expression is typical of the latest class porally regulated HSV genes (6). We are concerned here

with the mechanisms which underlie this observed temporally regulated pattern of expression.

In addition to US11 (6), late gene expression during virus infection has been studied using glycoprotein gC (7,8) and a late promoter (L42) identified by Hall et al.(9,10) as examples of prototype late genes. Under conditions of DNA synthesis inhibition gene products of US11 and gC fail to accumulate, but using sensitive assays are nevertheless detectable (6,8). In these experiments using chemical inhibitors it is not possible to conclude that absolutely no DNA replication had taken place, nor reach any firm conclusion concerning the mechanistic relationship between DNA replication and late gene expression.

To determine whether DNA synthesis is obligatory for late gene expression, the level of transcription from a plasmid-borne late promoter (US11) has been studied in the presence or absence of a functional HSV origin of DNA replication. The US11 promoter has been linked to the rabbit β-globin gene to distinguish US11 initiated RNA derived from the plasmid and the viral genome. After transfection of HeLa cells, which were then infected with HSV-1, the activity of the plasmid-borne US11 promoter was analysed by quantitation of correctly initiated RNA. The plasmid-borne US11 promoter was expressed with similar kinetics to the viral US11 promoter. The results indicate that DNA replication is not an absolute requirement for activation of the plasmid-borne US11 promoter, but is very important for achieving abundant expression. Transcription through the US11 promoter from an upstream promoter (belonging to the 3'co-terminal gene, IE-5) also appeared to play a positive role in achieving normal US11 gene expression.

MATERIALS AND METHODS

1. Cells and virus

HeLa cells used for transfections were grown in Dulbecco's modified Eagles medium supplemented with 2.5% calf serum and 2.5% foetal calf serum (Flow Laboratories). HSV-1 strain 17 syn^+ (11) was used for infection of transfected cells.

2. Plasmids

pRED4, pRED122 and pRED5 have been described (12,13).

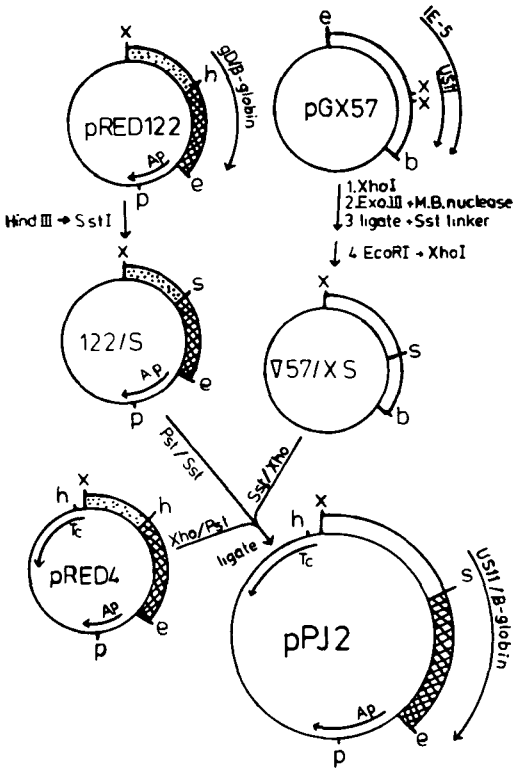

Figure 1. The Construction of pPJ2. HSV-1 DNA (-1269 to +37 of US11, open double lines) containing ORIS, IE-5 promoter and upstream activating sequence and the US11 capsites was derived from pGX57 via 57/X.S; the entire rabbit -globin gene (-7 to 400bp beyond the polyadenylation site, hatched box) from pRED.122 via 122/S; remaining pBR322 vector sequences (single line) including Tet^r gene from pRED4. pRED.122 and pRED4 contain gD promoter DNA (stippled box) from +11 to -33 and -396 respectively. Cloning steps were as indicated. Relevant restriction sites: e-EcoRI; x-XhoI; b-BamHI; h-HindIII; s-SstI; p-PstI.

pGX57 (5) contains most of the HSV-1 BamHI x fragment, bar a 135bp BamHI-EcoRI subfragment, and was supplied by F.Rixon (fig.1).

Construction of pPJ2: The essential features of pPJ2 are shown in figure 2.B. Briefly, its construction was as follows. The HSV portion (EcoRI-BamHI) of pGX57 was cut at its two internal XhoI sites, treated with exonuclease and religated with SstI linkers. The EcoRI site in the resulting plasmid was converted to XhoI, giving rise to the XhoI-SstI fragment of pPJ2. The HindIII linker at the gD/globin junction of pRED122 was converted to an SstI site, and the resulting SstI-PstI (from the PstI site in vector) fragment was used to provide the rabbit β-globin portion of pPJ2. Lastly, pBR322 vector sequences were completed with insertion of the appropriate XhoI-PstI fragment of pRED4. The HSV/β-globin junction of pPJ2 was sequenced to verify the final structure and ensure proximity to the US11

capsites (5,6). This construction is outlined in figure 1.

Construction of pPJ2-derivatives: As indicated in figure 2.B there are five (conveniently spaced) SmaI sites in pPJ2. A series of deletions were made by either; a) XhoI- partial SmaI digestion followed by filling in and religation (which reproduces a XhoI site),- pPJX series; or b) SstI-partial SmaI digestion followed by SalI linker ligation - pPJS series. Insertion of the appropriate XhoI-SalI fragments from the pPJS series into the XhoI site of pPJX5 gave rise to pPJ4, pPJ5 and pPJ8. pPJ9 was made simply by a partial SmaI digestion and religation. pPJ6 and pPJ7 were made by SmaI or SmaI-XhoI digestion respectively of pPJ4, followed by religation.

3. Calcium phosphate transfection, infection of HeLa cells, RNA isolation and S1 mapping analysis

Subconfluent layers of HeLa cells were transfected as described (12) using 10ug of the test plasmid and 10ug of the internal control plasmid, pRED5 (fig.2.A). Viral infections were performed 24h. after transfection and RNA prepared 16 hours after infection, except for the time-course experiment. The methods used for isolation and S1 mapping of RNA are described (12), using here as a probe the single stranded DNA fragment from pPJ2 shown in figure 2.B, labelled at the BstNI site, position +136 in the globin gene. This probe detects RNA transcripts initiated from the capsites of pPJ2 and pPJ2-derivatives. In addition, it detects RNA originating from the the internal control plasmid pRED5 (fig.2.A), resulting in S1 resistant bands which map to the break in homology at the gD/β-globin junction. Suitably exposed autoradiographs were analysed by densitometry (see 6). The ratio of correctly initiated 5'ends at the US11 promoter compared with bands from pRED5 corresponding to the homology break was calculated. The value of this ratio obtained with pPJ2 was taken to be 100 and ratios obtained with the mutants were expressed as a percentage of this. The activities given in figure 2.C are calculated from at least four independent experiments using two different plasmid preparations.

4. DNA isolation and analysis

DNA was isolated as described (6). One fifth of the total

DNA isolated for each time point was digested with SstI, PvuII and DpnI. The SstI/PvuII digestion allows discrimination between bands arising from pRED5 and pPJ2; DpnI cleaves the sequence GATC provided the A residue is methylated (by propagation of plasmids in dam$^+$ strains of E.coli). This provides the basis for a sensitive plasmid replication assay in eukaryotic cells (14). The digested DNA was analysed by Southern blot hybridization with pBR322 DNA ^{32}P-labelled in vitro (15,16). 1ng each of pRED5 and pPJ2 were treated as the cellular DNA extracts, with the omission of DpnI, to allow identification of bands corresponding to replicated pPJ2.

RESULTS

Construction and structure of pPJ2

The aim of this investigation was to study the effects of the presence of an HSV origin of replication on the activity of a plasmid-borne US11 promoter. The initial cloning objective was to construct a plasmid which directs properly regulated synthesis of correctly initiated transcripts from the US11 promoter, distinguishable from viral transcripts. This was based on the assumption that superinfection with HSV would be necessary to activate the plasmid-borne promoter in short-term transfection assays. Using the cloning scheme outlined in Materials and Methods and figure 1, plasmid pPJ2 was constructed. The region upstream from the US11 transcription start sites includes an origin of replication, and the promoter and upstream activating sequence of IE-5. Sequences which constitute the HSV-1 TR_S/IR_S origin of DNA replication (ORI_S) are contained within the region -785 to -825 relative to the US11 transcription start site at +1 (17). The promoter for IE-5 is contained within a region extending 69bp upstream from the IE-5 transcription start site (-640 relative to +1 of US11). The far-upstream sequence or enhancer-like element, which is required for an ~8 fold stimulation of the IE-5 promoter mediated by the virion component Vmw65 (18), is located on the far side of ORI_S relative to the IE-5 promoter; 944 to 1007 bp upstream from +1 of US11 (19). DNA sequencing confirmed the exact nucleotide sequence at the US11/β-globin junction. The

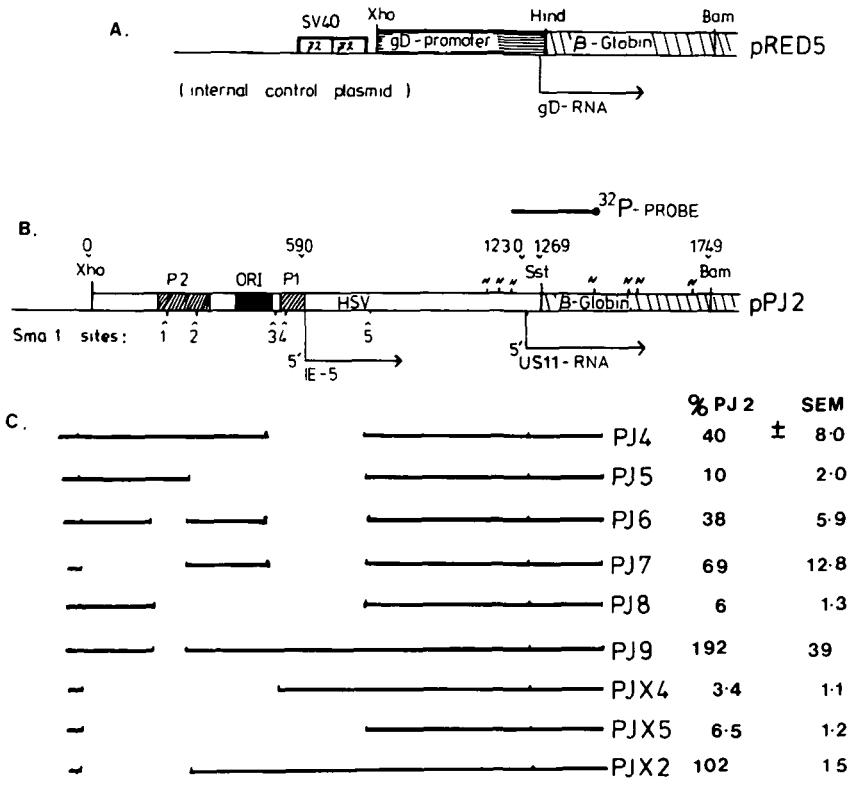

Figure 2. Detailed structures of pRED5, pPJ2 and pPJ-mutants. A. Internal control plasmid pRED5. gD promoter DNA (-396 to +11) is joined to the rabbit β-globin gene (-7 to beyond polyadenylation site) by a HindIII linker. SV40 DNA containing the 72bp repeat enhancer is directly upstream from the HSV gD DNA. B. pPJ2 with HSV-1 US11 DNA joined to rabbit β-globin DNA, shown aligned with pRED5, at an SstI linker. Indicated upstream from the US11 transcription start site: IE-5 transcription start site and distal promoter (P1); TR_S origin of replication (ORI); IE-5 upstream activating sequence (P2). The positions of relevant restriction sites are shown, including BstNI sites (N) used for probe DNA isolation, and 5 SmaI sites (1-5) employed for pPJ-mutant construction. Coordinates refer to bp from the XhoI site at 5'end of HSV DNA. Above pPJ2 is the single stranded DNA probe used to detect US11 initiated and pRED5 RNA, 5'end labelled at the BstNI site position +136 in the globin gene. C. Mutant test plasmids. Gaps represent deleted sequences, whose end points align to the numbered SmaI sites or the XhoI site. The identities of the constructs are on the right with their mean relative transcriptional activities after viral activation (pPJ2=100), and standard error of mean (SEM).

region 3' of +1 comprises 37 bp of untranslated US11 leader sequence, joined by an SstI linker to the entire rabbit β-globin gene, from -7 to 400 bp beyond the polyadenylation site.

Internal control plasmid: pRED5

Quantitation and comparison of the results obtained from independent experiments using different plasmid constructions requires the ability to standardise transfection and RNA detection efficiencies. For this purpose we used pRED5 as an internal control plasmid which produces β-globin transcripts from its gD promoter under cis-control of the SV40 enhancer (see fig.2.A). As described in Materials and Methods, the probe prepared from pPJ2 detects RNAs derived from pRED5 which span the break in homology between pPJ2 and pRED5. These RNAs, which include correctly initiated gD/β-globin RNA, varied in quantity during infection (fig.2.A), presumably in response to trans-acting viral factors which at early times increase gD expression from pRED5 above its cis-activated level mediated by the SV40 enhancer (13). At late times pRED5 RNAs were readily detectable (fig.3.A) and gave a reliable standardisation of the transfections irrespective of co-transfection with ORI^+ or ORI^- test plasmids (fig.4).

Time-course of appearance and accumulation of hybrid transcripts from the plasmid-borne US11 promoter

To investigate whether the plasmid-borne US11 promoter mimicked its viral counterpart during transfection experiments, US11/β-globin RNA accumulation and plasmid replication was analysed in HeLa cells which had been transfected with pPJ2 and superinfected with HSV for various lengths of time. Cytoplasmic RNA was probed for US11/β-globin hybrid transcripts by S1 mapping procedures, and the DNA analysis is described in the following section. The time-course experiment shown in figure 3.A included transfection with both pPJ2 and pRED5, except for the indicated controls. In the absence of viral infection, the US11 promoter in pPJ2 was inactive, which demonstrates the plasmid-borne promoter is dependent on virus induced trans-acting factors involved in transcription and replication for its activity. After activation, the pattern and location of the 5' transcriptional starts from the plasmid-borne US11

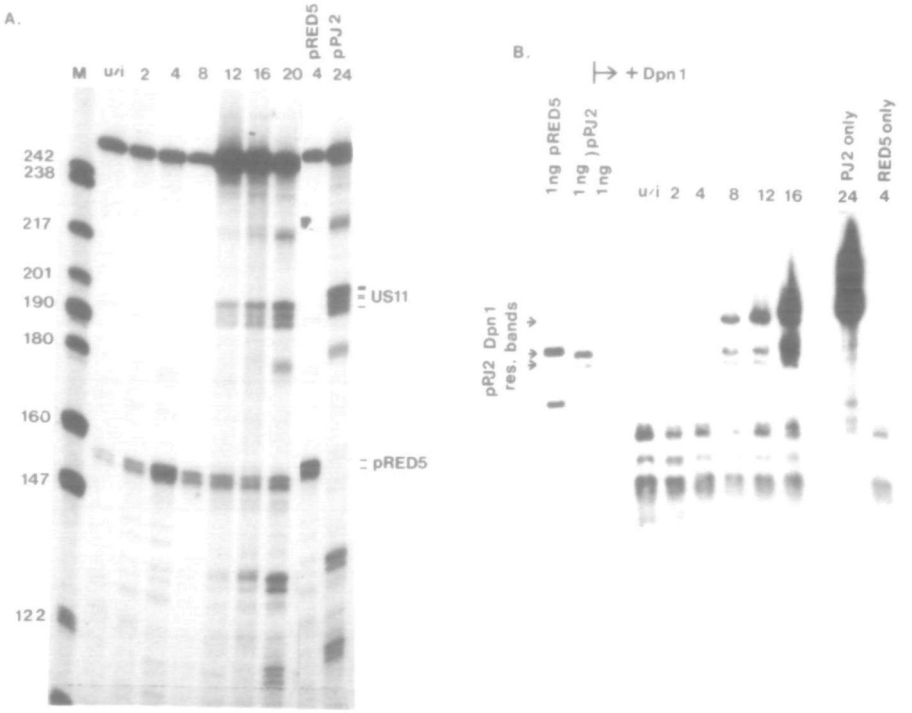

Figure 3. Time-course of plasmid-borne US11 promoter expression and replication of pPJ2. A. Nuclease S1 protected hybrids of RNAs after infection of HeLa cells transfected with pPJ2 and pRED5. Bands corresponding to US11 initiated hybrids and pRED5 RNAs which map to the pRED5/probe homology break are indicated. M=marker track (HpaII cut pBR322 DNA). B. DpnI assay of plasmid replication. Unlabelled DNA was prepared from the same cells as in A., and run on a 0.8% agarose gel following digestion. Blotted DNA was probed with ^{32}P-pBR322 DNA. The first 2 tracks correspond to 1ng of pRED5 or pPJ2 digested with SstI and PvuII only; all other tracks included DpnI in the digestion. Final 2 tracks of A. and B. are from single transfections of pPJ2 or pRED5.

promoter (fig.3.A) appeared to be identical to the transcriptional starts from the viral promoter (6). This was confirmed by running S1- protected hybrids of pPJ2 RNAs alongside a sequence ladder derived from the same probe (data not shown). The initial detection of hybrid gene transcripts in HeLa cells was surprisingly late at 8hrs (fig.3.A) compared to the appearance of viral US11 transcipts at 2hrs in BHK cells (6). We therefore examined US11 expression during viral

infection of HeLa cells. Viral US11 transcipts were not detectable before 6hrs p.a. and it was also noted that cytopathic effects were delayed compared to HSV-1 infection of BHK cells (data not shown). Thus in the same cell type, the plasmid-borne and analagous viral promoters are regulated with essentially the same kinetics of induction. The final two tracks in figure 3.A demonstrate the plasmid origin of the indicated US11 or pRED5 bands.

Replication of pPJ2

Analysis of the regulation of US11 in viral studies demonstrated the importance of viral DNA replication in achieving abundant gene expression (6). To test whether the origin of replication in pPJ2 was functional in transfected cells, DNA was extracted from the same cells used in the time-course experiment described above, and the samples analysed as described in Materials and Methods. Digestion with SstI and PvuII allows discrimination between bands arising from pRED5 and pPJ2. Treatment with DpnI distinguishes between replicated plasmid DNA and unreplicated input DNA of bacterial origin.

Figure 3.B shows the time-course of pPJ2 replication following superinfection with HSV-1. The appearance of DpnI resistant bands from pPJ2 clearly shows that the plasmid is efficiently replicated after infection of the transfected cells with HSV-1. At later times, concatemeric forms of pPJ2 become increasingly resistant to cleavage with SstI and PvuII due to partial digestion. There are no DpnI resistant bands derived from pRED5, indicating that, as expected, this plasmid is not replicated during the course of the transient assay, and that the DpnI digestion was complete in these samples.

Quantitation of US11 transcript and plasmid replication

The accumulation of US11 hybrid transcripts correlates well the replication of pPJ2 (fig.3.A,B), and is thus characteristic of normal late gene expression. Using the Southern blot shown in figure 3.B it was possible to estimate the number of plasmid copies per cell in the DNA samples of the time-course. The level of replicated plasmid molecules, which have necessarily spent some time in 'active areas' of the cell nucleus, should give a more reliable guide to template availability for

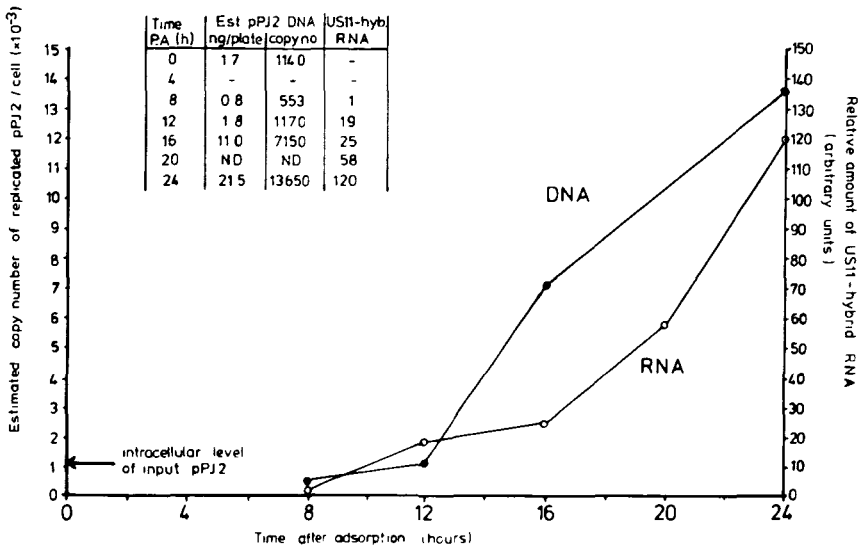

Figure 4. Accumulation of replicated pPJ2 and US11-hybrid transcripts in transfected cells after superinfection with HSV-1. The graph of DNA corresponds to the blot in fig.3.B. Copy number was estimated by calculating the ng equivalents of replicated pPJ2 present at each time point. On the assumption that 50% of transfected HeLa cells take up calcium phosphate precipitated DNA (based on unpublished immunoflouresence studies with the same cell line) 1ng pPJ2 ($1.3x10^8$ copies) = 650 copies per cell; 1/5th total DNA from $2x10^6$ cells was used for each analysis. The level of intracellular input pPJ2 was estimated from the DpnI digested DNA in uninfected cells, taking into account the proportion of pRED5 DNA. The relative amount of US11-hybrid transcript accumulation (from fig.3.A) takes the first detected level at 8hrs to be 1.

transcription than measurements of total isolated plasmid DNA. By comparing the relative rise in accumulated hybrid transcripts to replicated plasmid copy number, it was reasoned that information regarding the relationship between copy number and late gene expression could be derived.

Autoradiographs of the S1 gel shown in figure 3.A and a shorter exposure of the blot shown in figure 3.B (to ensure linearity between lighter and darker tracks) were scanned and quantitated. The estimated level of plasmid copies per cell and relative accumulation of US11-hybrid transcripts are shown in figure 4 (methods of calculation are described in fig.4 legend).

At 4hrs p.a. no replicated plasmid DNA was detectable, even on long exposures of the blot (the limit of sensitivity was estimated at approximately 4 copies/cell). Replicated pPJ2 DNA was first detected at 8hrs p.a., at a level of 530 copies per cell, and doubled between 8 and 12hrs p.a., and then increased 6-fold during the following 4hrs (fig.4). The input (unreplicated) plasmid DNA was estimated at 1100 copies of pPJ2 per cell (fig.4), which is consistent with the data of Alwine (20). Since DNA was extracted from the nuclei of transfected cells, it would seem reasonable to propose that most of the 1100 copies were intranuclear and were available for transcription and replication.

It was notable that US11-hybrid transcript accumulation dramatically increased (at least 10-fold) between 8 and 12hrs p.a., a period in which replicated template copy number had barely doubled (fig.4). Thereafter, transcript levels increased 1.3-fold between 12 and 16hrs, and in 2-fold steps up to 20 and 24 hrs. Hybrid transcript accumulation from 12hrs onwards increases rapidly, and thus resembles the increase in replicated template from 8hrs onwards, suggesting that transcript accumulation has increased proportionally with copy number during this phase. The implications of this are discussed later with consideration of the results from the following section.

Role of DNA replication and IE-5 transcription through the US11 promoter on US11 promoter activity

In order to investigate the absolute role of replication and possible effects caused by transcription from the IE-5 promoter on US11 gene expression, a series of derivatives of pPJ2 were constructed which contained deletions in the region far-upstream of the US11 RNA start sites. The construction of the pPJ2 derivatives, shown in figure 2.C, is outlined in Materials and Methods. US11 promoter activity was determined in *short-term transfection* assays by quantitative S1 *mapping of* RNA prepared 16h. after HSV-1 superinfection. Typical results obtained by S1 mapping are shown in figure 5 and the averaged data from at least four independent experiments using more than one preparation of each plasmid was determined.

The results in figure 2.C show that the pPJ2 derivatives

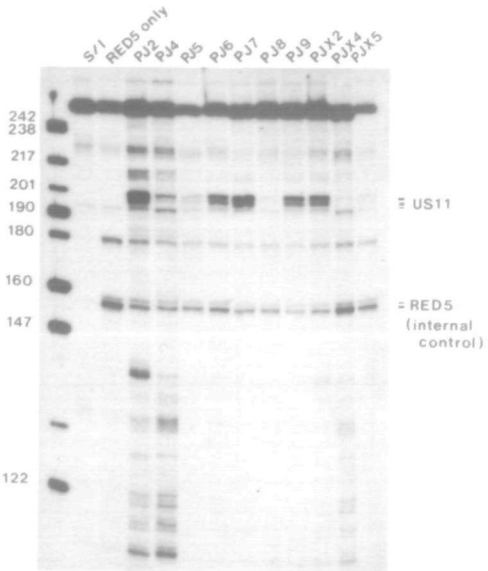

Figure 5. Comparison of transcriptional activation of pPJ-plasmids after superinfection of transfected cells with HSV-1 for 16 hrs. RNAs produced from the US11 hybrid gene and pRED5 are marked. Transfections included 10ug of indicated test plasmid and 10ug of pRED5 control plasmid, except for 2 tracks; S/I = RNA from only superinfected cells; RED5 only = no pPJ-test plasmid (shows that the background level of hybridization at the US11 position is negligible). Size standards are HpaII cut pBR322 DNA.

can be split into three groups, on the basis of a broad correlation between inducible promoter activity and far-upstream structure:

(i) The activities of pPJ5, pPJ8, pPJX4 and pPJX5 were consistently less than 10% of wild type pPJ2 activity, and their structures have in common the absence of ORI_S. The structure of pPJX4 differs from pPJX2 only by the absence of the origin containing sequence, yet the activity of the pPJX4-US11 promoter was reduced to 3-4% of pPJX2. These results clearly show that deletion of ORI_S results in a drastic decrease in US11 promoter activity. Analysis of DNA from transfected cells using the DpnI assay described earlier, showed a consistent correlation between ORI^+ plasmids and the appearance of replicated test plasmid DNA, whilst DpnI resistant bands were not observed in DNA samples where ORI^- plasmids had been tested (data not shown).

(ii) Plasmids which contain ORI_S but lack the IE-5 promoter (pPJ4, pPJ6 and pPJ7) have reduced US11 promoter activity compared to pPJ2, but substantially higher levels of expression compared to constructs lacking ORI_S. Since the IE-5 promoter is absent in pPJ4, 6 and 7, correctly initiated IE-5 RNA cannot be transcribed through the US11 promoter in these constructions.

These data indicate that such through-transcription may play a positive role in US11 transcription.

(iii) Plasmids which retain the IE-5 promoter and ORI_S have similar levels of expression compared to pPJ2, as seen with pPJX2, or substantially higher, in the case of pPJ9. The result with pPJ9 suggested that the presence of the IE-5 far-upstream activating sequence may be acting negatively on US11 promoter activity, but this hypothesis was inconsistent with the results for pPJX2. Similar comparison between the activities and structures of pPJ4, pPJ6 and pPJ7 yielded no obvious role for the far upstream element of IE-5 in moderating US11 promoter activity.

DISCUSSION

This report describes the use of a short-term transfection assay to study the regulated activity of an HSV-1 late promoter by quantitative S1 mapping. The behaviour of the plasmid-borne US11 promoter closely mimicked its viral counterpart during productive infection. In response to activation during HSV-1 infection, correctly initiated transcripts were produced from the plasmid copy of US11 with the same kinetics as viral US11 transcripts, and similarly, their appearance coincided with the onset of DNA replication. Hybrid transcripts were not detected in mock-infected cells, indicating that induction of the plasmid-borne US11 promoter, like its viral counterpart, requires viral gene products.

An important question is the validity of extrapolating findings from a transient assay to the viral situation. Several lines of evidence suggest that promoter regulation in a transient assay is representative of viral gene regulation. The intact plasmid-borne US11 (in this study) and gD (12) promoters behaved in transient assays like their viral counterparts during viral infection, and a series of mutations in the tk promoter upstream of the mRNA cap site exerted qualitatively similar effects in both transient assays and reconstructed viral gemomes (21; D. Coen, S. Weinheimer, L. Leslie, and S. McKnight, personal communication). In addition, both plasmid and viral DNA molecules are in open chromatin structure in the nucleus

(22,23), which suggests that their physical state may be similar. The >95% reduction in US11 initiated transcript accumulation from replication-inhibited viral genomes (6) and ORI⁻ plasmids (fig.2.C -with the exception of pPJ5) is strikingly similar. Allowing for a particle/pfu ratio of 20-50 in our HSV-1 stocks the number of viral genomes entering a cell at an m.o.i. of 20 pfu (6) is unlikely to exceed the 500-1000 intracellular plasmid copies estimated in this study. This comparison suggests that plasmid and viral templates are transcribed with similar poor efficiency in the absence of replication. A particularly good transfection efficiency and sensitive expression assay (e.g. CAT-assays) could account for a seemingly high level of late gene expression in the absence of DNA replication in some experimental systems (24).

In previous studies using phosphonoacetic acid (PAA) to inhibit DNA replication, viral US11 gene expression was reduced 50-100 fold (6). However, in the viral experiments it was not clear whether the low level of US11 gene expression detected in the presence of PAA was due to incomplete inhibition of DNA synthesis or real replication-independent expression. The transient transfection assay system has demonstrated that the US11 promoter is functional in the absence of an origin of DNA replication. However, replication plays a major role in achieving abundant expression since US11 transcription from ORI⁻ plasmids was reduced 10-25 fold compared to the 'wild-type' ORI⁺ plasmid. True-late gene expression under non-replicating conditions has been recently reported for gC using wild-type virus and a ts DNA⁻ mutant at the non-permissive temperature, both in the presence of PAA (8). DeLuca and Schaffer (24) have recently shown that the L42 late promoter identified by Hall et.al., (9) when linked to the CAT gene on a ORI⁻ plasmid is inducible by superinfection with HSV. Thus there is increasing evidence that activation of late gene expression does not require DNA replication a priori.

Replication of template DNA is necessary for abundant late gene expression. This may be achieved through increased copy number, and/or an altered template structure during or after replication of the DNA. An obvious role for replication would

be to increase the copy number of an intrinsically weak promoter. Since late genes can be activated in the absence of replication, it is plausible that the mechanism of their activation may be analagous to early genes, except that late promoters are comparibly poorer and thus are more dependent on high copy number for abundant expression. This has been shown to be the case for the SV40 late promoter (25,26). Since HSV early gene products do not accumulate in parallel with those of late genes after replication, the early promoter may be negatively regulated at late times, or they may compete for limited transcriptional factors not required by late genes or their gene products may become less stable.

The data in figure 4 indicates that the role of replication in late gene transcription may be more comlex than merely to increase copy number. Before the onset of DNA replication, up to 1000 copies per cell of plasmid DNA were isolated from transfected cell nuclei, yet no US11 transcription was detectable. US11 transcripts became detectable when there were 500 copies per cell of replicated plasmid DNA. The similarity in the times of appearance of US11 hybrid transcripts and pPJ2 plasmid DNA replication is striking. This observation suggests that template replication per se may account for an increase in late promoter activity, and thereafter transcript accumulation follows replicated DNA copy number (fig.4). However there is no evidence to distinguish between a lasting change in replicated structure from the transient act of replication itself in affecting late promoter activity. Speculatively, replication of and transcription through the US11 promoter (from the IE-5 promoter) may indicate a common mechanism by which both processes operate positively on late gene expression; both would lead to an opening up of the template structure to improve access for transcription machinery. According to this hypothesis the actual rate of late gene expression should drop immediately after replication has been inhibited late in infection. We are planning to test this idea.

The mechanism of late gene activation and the structural features that distinguish a late promoter from those of earlier classes remains unclear. Recently published evidence (24,27)

suggests that the trans-acting requirements for late gene activation may differ from earlier classes. We are currently investigating the sequence requirements for activation of the US11 promoter.

In summary, the activation of the US11 promoter is not dependent on, but substantially increased by DNA replication. The involvement of DNA replication in late gene expression has accounted for the placing of these promoters in a different class to other HSV genes, on the reasonable grounds of their characteristic expression kinetics. More detailed analysis of the rate of late gene expression during the presence and absence of DNA synthesis should aid the understanding of the link between DNA replication and late gene expression. Investigation of the cis-DNA sequences necessary for activation of a late promoter will yield clues concerning its trans-acting requirements and aid a more precise definition of late promoter.

Acknowledgements

We are grateful to Frazer Rixon for pGX57, and Mark Harris and Ron Hay for advice with the *DpnI* assay. Many thanks to Nigel Stow and John Subak-Sharpe for critical advice with the manuscript. PAJ was supported by a SERC studentship.

REFERENCES

1. Honess, R.W. and Roizman, B. (1974) J. Virol. 14, 8-19.
2. Clements, J.B., Watson, R.J. and Wilkie, N.M. (1977) Cell 12, 275-285.
3. Jones, P.C. and Roizman, B. (1979) J.Virol. 31, 299-314.
4. McGeoch, D.J., Dolan, A., Donald, S. and Rixon, F.J. (1985) J. Mol. Biol. 181, 1-13.
5. Rixon, F.J. and McGeoch, D.J. (1984) Nucl. Acids Res. 12, 2473-2487.
6. Johnson, P.A., MacLean, C., Marsden, H.S., Dalziel, R.G. and Everett, R.D. (in press) J. Gen. Virol.
7. Johnson, D.C. and Spear, P.G. (1984) J. Virol. 51, 389-394.
8. Godowski, P.J. and Knipe, D.M. (1985) J. Virol. 55, 357-365.
9. Hall, L.M., Draper, K.G., Frink, R.J., Costa, R.H. and Wagner, E.K. (1982) J. Virol. 43, 594-607.
10. Silver, S. and Roizman, B.R. (1985) Mol. Cell. Biol. 5, 518-528.
11. Brown, S.M., Ritchie, D.A. and Subak-Sharpe, J.H. (1973) J. Gen. Virol. 18, 329-346.
12. Everett, R.D. (1983) Nucl. Acids Res. 11, 6647-6666.
13. Everett, R.D. (1984a) Nucl. Acids Res. 12, 3037-3056.
14. Stow, N.D. (1985) J. Gen. Virol. 66, 31-42.
15. Southern, E.M. (1975) J. Mol. Biol. 98, 503-517.
16. Rigby, P.W.J., Dickman, M., Rhodes, C. and Berg, P. (1977) J. Mol. Biol. 113, 237-251.

17. Stow, N.D. and McMonagle, E.C. (1983) Virology 130, 427-438.
18. Campbell, M.E.M., Palfreyman, J.W. and Preston, C.M. (1984) J. Mol. Biol. 180, 1-19.
19. Preston, C.M., Cordingley, M.G. and Stow, N.D. (1984) J. Virol. 50, 708-716.
20. Alwine, J.C. (1985) Mol. Cell. Biol. 5, 1034-1042.
21. Eisenberg, S.P., Coen, D.M. and McKnight, S.L. (1985) Mol. Cell. Biol. 6, 1940-1947.
22. Leinbach, S.S. and Summers, W.C. (1980) J. Gen. Virol. 51, 45-59.
23. Cereghini, S. and Yaniv, M. (1984) EMBO J. 3, 1243-1253.
24. DeLuca, N.A. and Schaffer, P.A. (1985) Mol. Cell. Biol. 5, 1997-2008.
25. Keller, J.M. and Alwine, J.C. (1984) Cell 36, 381-389.
26. Hartzell, S.W., Byrne, B.J. and Subramanian, K.N. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 23-27.
27. Sacks, W.R., Greene, C.C., Aschamn, D.P. and Schaffer, P.A. (1985) J. Virol. 3, 796-805.