Transcriptional induction of the ubiquitin gene during herpes simplex virus infection is dependent upon the viral immediate-early protein ICP4

D.S.Latchman, J.K.Estridge and L.M.Kemp

Department of Zoology and Cell Biology, University College, London WC1E 6BT, UK

ABSTRACT
    Lytic infection with herpes simplex virus results in transcriptional induction of a cellular gene encoding ubiquitin, causing increased accumulation of ubiquitin RNA and protein in the infected cell. This induction, which is dependent upon viral protein synthesis, does not occur in the HSV-1 mutant tsK which is defective in the gene encoding the viral protein ICP4. Transfected cells expressing the viral ICP4 protein exhibit higher levels of ubiquitin gene transcription than untransfected controls indicating that transcriptional induction can be mediated by the ICP4 protein alone.

INTRODUCTION
    Lytic infection with herpes simplex viruses (HSV) types 1 and 2 causes a well characterised shut off of cellular protein synthesis (reviewed in 1) which is mediated at a number of levels including the disagregation of polysomes synthesizing cellular proteins (2,3) and the degradation of cellular RNA species (4,5). Despite this a small number of cellular proteins increase in abundance upon infection (6,7) and in at least one case this is paralleled by a transcriptional induction of the corresponding cellular gene (8). Interestingly, at least two of these proteins are also over-expressed in cells transformed by HSV or other agents (9), a finding of particular importance in view of the suggestion that HSV transforms via a hit and run mechanism in which transient exposure to the virus produces a permanent effect on the host cell (10).
In order to identify other genes and proteins activated by HSV infection and investigate the mechanism of this effect we have used differential screening techniques to isolate cDNA clones derived from cellular genes transcriptionally induced by infection with HSV (11). Here we report the identification of one of these genes as encoding ubiquitin, a heat inducible 76 amino acid polypeptide which is attached to proteins that are substrates for proteolytic degradation (12,13). We also show that the transcriptional

induction of this gene in HSV infection is mediated by the product of the viral ICP4 gene.

MATERIALS AND METHODS
Cells and Viruses
Baby hamster kidney cells (BHK) clone 13 (14) were grown in Eagle's medium supplemented with 10% new born calf serum and were infected with HSV-2 strain 333 at a multiplicity of 5pfu/cell. For infections in the presence of cycloheximide (200μg/ml) the drug was added thirty minutes prior to viral infection and remained present until cells were harvested. For infections in the presence of acyclovir, the drug was added at the time of infection and remained present until cells were harvested. In both cases mock infected cells were similarly treated without addition of virus. Infection with the HSV-1 deletion mutant dll403 (15) was carried out exactly as for wild type virus. Infections with the temperature sensitive mutants of HSV-1, tsK (16) and tsY46 (17) were carried out at the non-permissive temperature (38.5°C and 39°C respectively), mock infected cells being maintained at similar temperatures. In all cases cells were harvested seven hours after infection. LTA and Z4 cells were grown as described by Persson et al (18).
Northern Blotting
Northern blotting was carried out as previously described (11) using RNA samples equalised both by hybridisation with ^{3}H poly U as previously described (19) and by prior hybridisation with a cDNA clone derived from an mRNA whose level does not change upon infection with HSV-2 (11). Blots were hybridizd with a cDNA clone derived from the human UbB gene (pRBL26- reference 20). This clone was also used in the nuclear run-off assays which were carried out as previously described (11).
### Immunfluorescence
Internal staining was carried out as described by La Thangue et al (6) using monoclonal antibody D83 to ubiquitin (a kind gift of Dan Finley and Alexander Varshavsky) as the first layer and rhodamine-conjugated goat anti-mouse immunoglobulin as the second layer.
Plaque screening
Stocks of recombinant phage in the vector λ gt10 were spotted onto a lawn of growing E.coli and hybridized as previously described (11).

RESULTS

We have previously reported (11) the use of differential screening techniques to isolate cDNA clones derived from cellular mRNAs up-regulated upon HSV-2 infection of BHK cells. In view of the accumulation of heat shock proteins which occurs during HSV infection of such cells (6,21) we hybridized these clones with cDNA probes derived from several heat-inducible genes. In this experiment (figure 1) two clones showed strong reactivity with a probe derived from a human ubiquitin gene (UbB:- 20). Northern blotting with this probe confirmed that an increase in abundance of ubiquitin RNA occurs in HSV-2 infection (figure 2a and b, tracks 1 and 2), a transcript of 1.7kb increasing approximately five fold in abundance, whilst transcripts of 3.2 kb and 650 bases were unaffected or marginally decreased. The sizes of
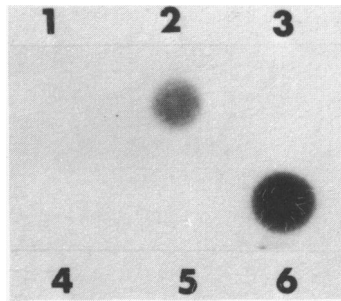
Figure 1.   Hybridization of plaques derived from up-regulated cDNA clones
(1-6) with the ubiquitin cDNA clone pRBL26.

these transcripts, obtained by comparison with the mobilities of the ribosomal
RNAs and of the human ubiquitin RNAs whose sizes are well characterised
(22,23), agree well with those previously reported for the three ubiquitin RNA
species detected in rat cells (23).
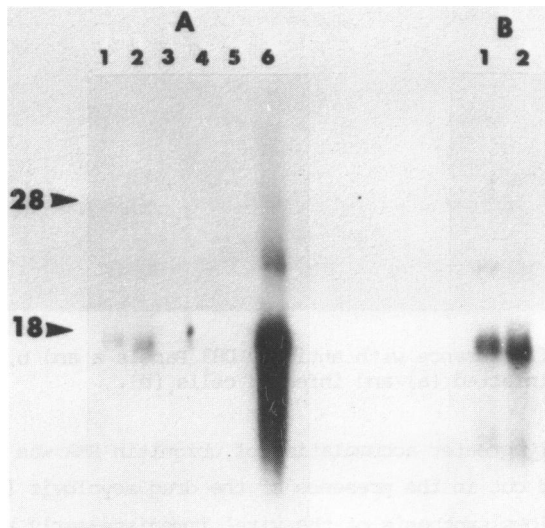
Figure 2.   Northern blot of mRNA samples with the ubiquitin cDNA clone
pRBL26.   Tracks 1, 3 and 5 mock infected BHK cells tracks 2, 4 and 6.   BHK
cells infected with HSV-2.   Samples in tracks 3 and 4 were prepared from cells
mock infected or infected in the presence of cycloheximide (200µg/ml), those
in tracks 5 and 6 from cells mock infected or infected in the presence of
acyclovir (200uM).   Panels a and b represent different exposures of the same
blot, that in a being exposed for 6 hours whilst that in b was exposed for 40
hours.   Arrows indicate the positions of ribosomal RNA markers.
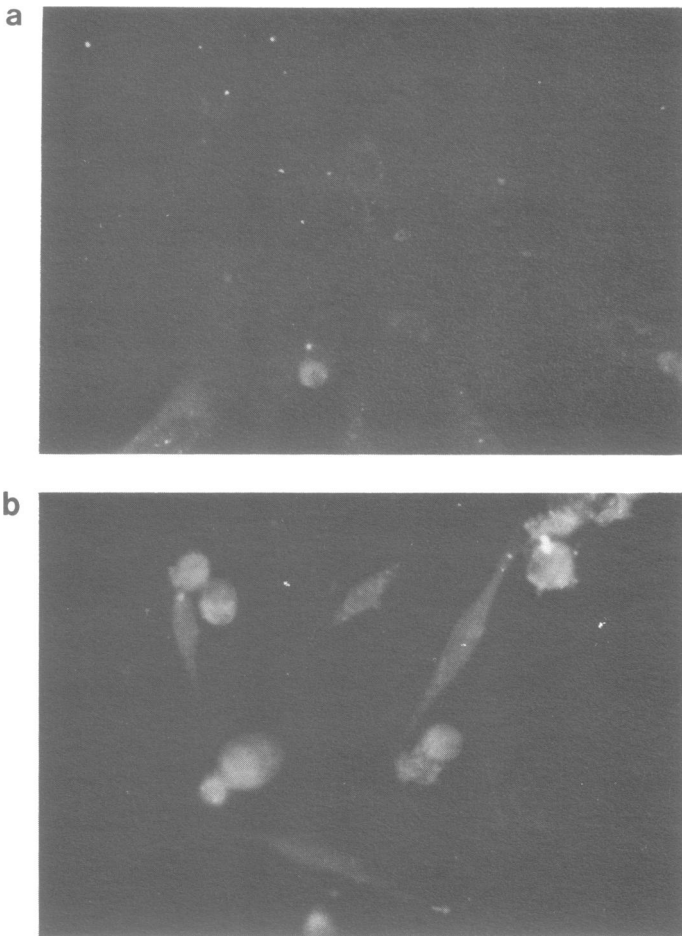
Figure 3.   Immunfluoresence with antibody D83 Panels a and b, internal staining of mock infected (a) and infected cells (b).

A considerably greater accumulation of ubiquitin RNA was observed during infections carried out in the presence of the drug acyclovir (figure 2a,tracks 5 and 6) which allows synthesis of the viral immediate-early and early proteins to occur but inhibits viral DNA replication and hence the replication dependent accumulation of the viral late proteins and subsequent cell lysis (24). Thus the accumulation of ubiquitin RNA does not require such replication and indeed the accumulation observable in normal infections appears to be limited by events occuring during or subsequent to such replication, presumably progressive cytolysis and cell death. In contrast no

Table 1.
Result of nuclear run-off assays with infected and mock-infected cells.

	Mock Infected	HSV-2 infected
Ubiquitin	20	75
clone 95	65	35
clone 123	120	94

Figures are counts per minute binding to 5µg of the indicated clone in
hybridizations with RNA synthesized by the various nuclei under run-off
conditions. Figures are the average of two determinations, the
background obtained with plasmid vector (approximately 10cpm) has been
substracted in each case. Clones 95 and 123 contain inserts derived from
mRNA species where levels do not change in infection with HSV-2 (11).

accumulation of ubiquitin RNA was observed when cells infected in the presence
of cycloheximide to prevent viral protein synthesis were compared to similarly
treated mock-infected cells, indicating that at least one viral protein must
be synthsised in the infected cell for this effect to occur (figure 2a, tracks
3 and 4). Interestingly, the levels of ubiquitin RNA were generally reduced
in cells treated with cycloheximide, with or without added virus, (compare
figure 2a tracks 1 and 3) suggesting that cycloheximide itself may have a
repressive effect on ubiquitin gene expression.

In view of the fact that increased accumulation of the ubiquitin protein,
itself has not previously been reported in virally infected cells, we used
indirect immunofluoresence with a monoclonal antibody to this protein to
investigate whether the increased ubiquitin RNA level in HSV-2 infected cells
resulted in increased protein accumulation. In these experiments (figure 3)
such increased ubiquitin levels were readily detectable in the infected
cells. Similar results were obtained when cells were infected in the presence

Table 2. Results of nuclear run-off assays with mutants in viral immediate-
early genes.

Temperature	37°C	37°C	38.5°C	38.5°C	39°C	39°C
Infection	Mock	dl1403	Mock	tsK	Mock	tsY46
IE affected	–	ICP0	–	ICP4	–	ICP27
Ubiquitin	25	75	34	20	45	92
95	85	58	92	54	91	51
123	110	74	145	71	115	83

Figures are counts per minute binding to the indicated clone in
hybridisations with RNA synthesized by the various nuclei and were obtained
as described in the legend to Table 1.

Table 3   Results of nuclear-run off assays

	LTA	Z4 cells
Ubiquitin	25	83
95	74	70
123	112	114

Figures are counts per minute binding to the indicated clone in
hybridisation with RNA synthesised by the various nuclei and were obtained
as described in the legend to Table 1.

of acyclovir (not shown). The increased staining in infected cells was
specific to the anti-ubiquitin antibody, no such increase being observed with
antibodies to other cellular proteins.

        To investigate further the processes mediating the observed accumulation
of ubiquitin RNA and protein we used nuclear run-off assays to compare the
rate of transcription of the ubiquitin gene(s) in infected and mock infected
cells. In these experiments (table 1) a clear increase in transcription was
detectable in the infected cells whilst the transcription rate of genes
encoding RNA species whose level does not change upon infection (11) was
reduced, a phenomenon we have discussed in more detail elsewhere (Kemp and
Latchman submitted). A similar increase in transcription was detectable in
cells infected in the presence of acyclovir but not in those infected in
the presence of cycloheximide (not shown). Thus the observed increases in
ubiquitin RNA and protein levels, are mediated at least in part by
transcriptional induction of the corresponding gene(s) which is dependent upon
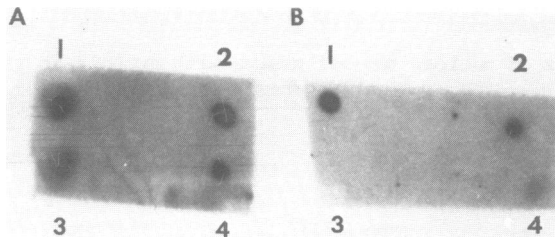the synthesis of viral protein(s) in the infected cell.

Figure 4.   Nuclear run-off assay using nuclei prepared from Z4 (A) and LTA
(B) cells. 1 and 2, 5µg DNA from control clones (95 and 123 respectively)
derived from mRNA species whose level does not change upon HSV infection. 3
and 4, 1µg and 5µg DNA respectively of the ubiquitin cDNA clone pRBL26.

The obvious candidates for mediating such induction, are the three viral immediate-early proteins, ICP0, ICP4 and ICP27, which have been shown to have trans-activating ability in co-transfection experiments (25) and at least two of which (ICP4 and ICP27) are indispensable for activation of the early and late stages of viral gene expression during lytic infection (16,17). We therefore carried out infections with viral strains carrying mutations in each of the genes encoding these proteins and studied the effect on the transcription of the ubiquitin gene(s). In these experiments (table 2) up-regulation of ubiquitin transcription was not observed during infections with the HSV-1 mutant tsK which carries a mutation in the gene encoding ICP4 (16) whilst such induction was observed with the other mutants. A similar failure to induce increased transcription of the ubiquitin gene was also observed with another mutant in the HSV-1 ICP4 gene, tsLB2 (26) (not shown). These findings indicate that induction of the ubiquitin genes is dependent upon a functional ICP4 protein. This protein is also required, however, for the induction of the viral early genes which does not occur in infections with tsK or tsLB2. Hence this experiment does not allow an assessment of whether the effect of ICP4 on the ubiquitin genes is a direct one or is mediated indirectly via activation of a viral early gene. In order to investigate this effect further, we made use of the cell line Z4 into which a 10kb fragment of HSV-1 DNA containing the complete ICP4 gene has been stably introduced (18) and which expresses high levels of the ICP4 gene product. This DNA fragment does not contain the complete transcription unit of any other gene, although it does contain the coding sequence of the immediate-early protein ICP47. The small amount of this protein made in these cells is unlikely to affect our results in that a viral strain in which this gene has been completely deleted (27) shows no difference from the wild type in the induction of the ubiquitin gene (not shown).In experiments comparing the levels of ubiquitin gene transcription in Z4 cells with that in its untransfected parent (LTA) increased transcription was detectable in the stably transformed cell (table 3 and Figure 4) indicating that the induction of the ubiquitin gene is dependent upon the ICP4 protein itself and can occur in the absence of other viral gene productsthe first time such an effect has been described for an HSV gene product and a cellular gene.

DISCUSSION

Lytic infection with HSV results in the activation of a small number of cellular genes (8,11). Although a minority of these genes can be activated

prior to viral protein synthesis (28), the majority are dependent upon such synthesis for their activation (8,28). Here we show that in at least one case, that of the ubiquitin gene, such a requirement for the synthesis of viral proteins can be fulfilled by the product of the viral ICP4 gene alone. The ability of the ICP4 protein to trans-activate the ubiquitin gene parallels its essential role in the activation of the viral early genes (18,29) and its ability to trans-activate these genes in co-transfection experiments (30,31). The ability of this viral protein to activate both viral and some cellular genes is paralleled in other DNA tumour viruses such as SV40 and adenovirus where the proteins whose trans-activating ability is of major importance for the viral lytic cycle also induce a small number of cellular genes (32,33,34). Such a property may thus be a general and important one of the first proteins synthesized in cells during lytic and abortive infections with the DNA tumour viruses. Indeed it has been suggested that the ability to induce cellular gene expression may be involved in the ability of these viruses to transform cells which are non-permissive for lytic infection (35).

In the case of HSV, another immediate-early protein, the ICP0 protein can also activate viral and cellular promoters in co-transfection experiments (25,30,36) and in concert with ICP4 has been shown to induce integrated cellular promoters recently introduced into cultured cells by transfection, although the endogenous gene within its normal chromatin structure is not affected (37). In our experiments however, no effect on the induction of the ubiquitin gene was observed when a strain carrying a deletion in the ICP0 gene (15) was compared to wild type virus. In this regard it is of interest that, unlike ICP4, functional ICP0 does not appear to be indispensable for lytic infection. Thus, although mutants lacking the gene encoding this protein grow poorly, especially at low multiplicities of infection, they are capable of expressing the viral early and late genes and of a complete lytic cycle (15, 38). It seems likely therefore that despite its trans-activating ability in co-transfection experiments this protein does not play an essential role in the activation of viral and cellular genes in lytic infection and that its primary role may lie elsewhere perhaps in the regulation of latent infections (36).

In contrast functional ICP4 protein is essential for the induction both of the viral early genes and of that encoding ubiquitin. In our experiments the up-regulation of the ubiquitin gene in Z4 cells producing ICP4 was specific to this gene, other cellular genes having the same transcription rate

in these cells as in the untransfected parent (LTA). Similarly, of the three ubiquitin RNA species, which in humans are derived from different genes (20,22), only one was dramatically induced during infection (fig. 2). Such findings suggest that common sequences mediating ICP4-dependent induction may exist in the viral early gene promoters and that of one member of the ubiquitin gene family. The lack of available sequence information for the rodent ubiquitin genes prevents an assessment of whether this is the case. However, no such virus specific DNA sequence regulatory element has been detected in the viral early promoters (39,40) and the mechanism by which ICP4 induces viral and a few cellular genes remains obscure.

Although up-regulation of the ubiquitin gene during viral infection has not previously been noted, it is known to be induced in heat shock (13). This induction has been suggested to be due to an accumulation of heat denatured proteins in the stressed cell (41), such proteins becoming conjugated to ubiquitin and hence being marked for degradation by the ubiquitin-dependent degradation system (12). It is probable that the induction of ubiquitin in HSV infection represents a similar attempt by the cell to deal with the presence of abnormal viral proteins, although such induction is mediated via ICP4 dependent induction of the ubiquitin gene and does not occur for example in response to the entry of the HSV alone. Such a possibility is supported by the finding that HSV infection does not induce accumulation of the 650 bp ubiquitin mRNA species which has been shown in both human and rat to encode a nuclear form of ubiquitin that is stably conjugated to histone H2A and is not involved in proteolytic degradation (23,42). It is likely therefore that the induction of ubiquitin in HSV infection represents one aspect of a co-ordinate induction of cellular degradative pathways which also results in the accumulation of a protein homologous to the Lon protease of E.coli, a protein which is involved in the degradation of abnormal or denatured proteins (43,44,45). Although in cultured cells, such attempts to inhibit the progress of the viral lytic cycle are usually unsuccessful, it is possible that during infection of some cell types in vivo, they may cause the lytic cycle to be aborted, resulting in either latent infection (46) or possibly cellular transformation (47) by HSV. We are currently investigating the expression of the ubiquitin gene in cell types latently infected or transformed by HSV in order to explore this possibility further.

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