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Derepression of a novel class of vaccinia virus genes upon DNA replication

Jan C.Vos and Hendrik G.Stunnenberg

European Molecular Biology Laboratory, Meyerhofstrasse 1, 6900 Heidelberg, FRG

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A novel class of vaccinia virus genes, called intermediate, is expressed immediately post-replication and prior to the onset of late gene transcription. Intermediate transcription is dependent on *trans*-acting factors which are present in an active state in virus-infected cells prior to the onset of DNA replication. Plasmid-borne intermediate genes transfected into vaccinia-virus infected cells are expressed prior to DNA replication, whereas the copies within the viral genome are repressed. DNA replication is essential for activation of viral intermediate transcription and *de novo* protein synthesis is not required post-replication. In contrast, activation of late transcription depends on DNA replication and continued *de novo* protein synthesis. Therefore, a subset of intermediate proteins is likely to be *trans*-activators of late gene transcription. Cell-free extracts differentially transcribe early, intermediate and late genes in a way similar to the temporal expression observed *in vivo*. A cascade model is discussed for the regulation of gene expression during the viral life-cycle.

Key words: vaccinia virus/intermediate genes/*in vitro* transcription/transient expression/derepression

Introduction

DNA replication plays an important role in the regulation of transcription during development and differentiation. It has been shown that DNA replication is required for the onset of repression of silent mating-type loci in yeast, whereas derepression can occur in the absence of replication (Miller and Nasmyth, 1984). The effects of replication on transcription of 5S genes *in vitro* is reported to be due to the movement of the replication fork through the transcriptional control region, thereby disassembling the committed transcription complex (Wolffe and Brown, 1986). Replication-dependent switching in gene expression is a commonly observed regulatory control mechanism in nuclear viruses, e.g. herpes simplex virus (HSV), adenovirus (Ad) and simian virus 40 (SV40) (for review see Tooze, 1981). Several years ago, Thomas and Mathews (1980) showed, using a superinfection protocol, that replication of the viral template *per se* is required for expression of late regions in the Ad genome. However, the elucidation of the molecular mechanisms underlying the replication-dependent repression or derepression of gene expression has been hampered by the lack of an *in vitro* transcription system which faithfully reflects *in vivo* patterns of expression. Viral promoters, such as the Ad major late, are faithfully transcribed in *in vitro* cell-free transcription systems derived from uninfected cells (Manley *et al.*, 1980). Furthermore, *cis*-acting regions involved in switching often overlap origins of replication, complicating the molecular analyses (DePamphilis, 1988).

Vaccinia virus may be an ideal model system to study replication-dependent control of gene expression. The regulation of viral transcription and replication is probably independent of cellular components; most (if not all) of the enzymes and factors are encoded in the viral genome. Furthermore, discrete origins of replication have not been identified in the viral genome. In fact, bacterial plasmids are replicated when introduced into poxvirus-infected cells (DeLange and McFadden, 1986).

Productive infections with vaccinia virus proceed through two defined phases, early and late, delineated by the onset of virus DNA replication and characterized by the expression of different subsets of genes which are scattered over the viral genome. The transcription of early genes, starting immediately after infection, is independent of *de novo* protein synthesis; the enzymes and factors required for early transcription are contained within the virus particle (Wei and Moss, 1974; Baroudy and Moss, 1980). Late transcription is dependent on DNA replication and early viral products are assumed to be required. Late mRNAs transcribed from the class of genes containing the conserved and essential TAAAT motif at the site of transcription initiation are distinguishable from early transcripts because of read-through of the RNA polymerase in the late phase of infection resulting in heterogeneous 3′ termini (Moss, 1985). These transcripts are further characterized by the presence of a 5′-terminal poly(A) stretch, the poly(A) head, which is not encoded in the genome (Patel and Pickup, 1987; Schwer *et al.*, 1987; Wright and Moss, 1987). This poly(A) head is probably generated by slippage (stuttering) of the polymerase complex during transcription initiation (DeMagistris and Stunnenberg, 1988; Schwer and Stunnenberg, 1988).

We have investigated the phenomenon of replication-dependent activation of vaccinia genes by analysing transcription from several different promoters using a transfection assay. We have identified a novel class of post-replicative genes which we will refer to as intermediate, based on the time of activation of these genes during the infectious cycle. Finally, we have established a cell-free transcription system which specifically recognizes intermediate promoters.

Results

We have investigated the temporal expression of vaccinia virus genes by transfecting plasmids containing different viral promoters into infected cells and monitoring the level of transcription at different stages of infection. The vaccinia promoters (Figure 1) used in these experiments have only been partially characterized as to the time of their activation during the infectious cycle. The promoter of the 11-kd

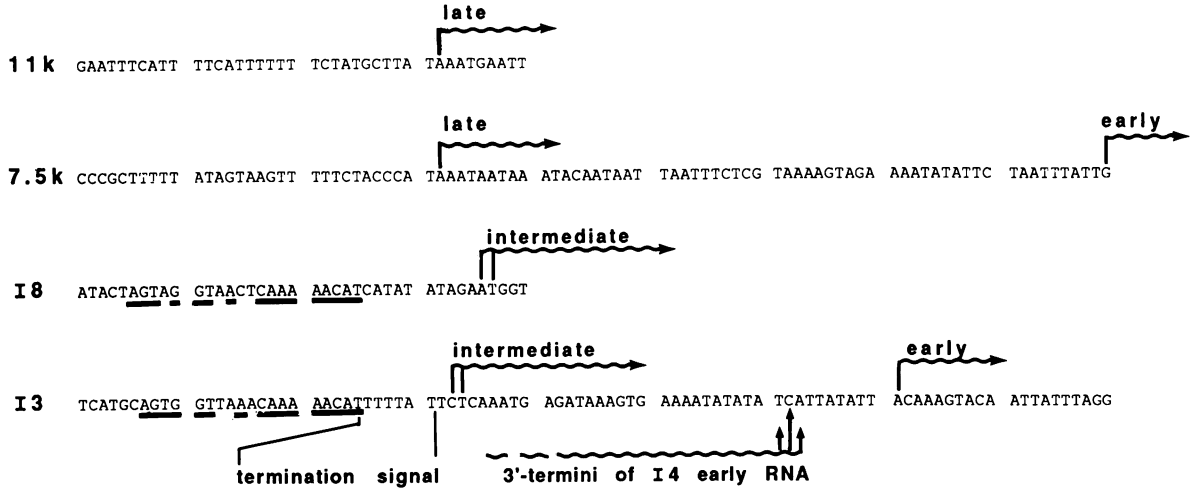

Fig. 1. Promoter sequences. The sequence and RNA start sites (indicated by arrows) were determined by Bertholet *et al.* (1985), Cochran *et al.* (1985b), Schmitt and Stunnenberg (1988) for 11K, 7.5K, I8 and I3 respectively. The sequence homology between the intermediate I3 and I8 promoters is underlined.

basic polypeptide is transcribed exclusively in the late phase of infection (Bertholet *et al.*, 1985; Hänggi *et al.*, 1986). This promoter can be regarded as a typical late promoter, having the conserved and essential TAAAT sequence motif at the site of transcription initiation (Hänggi *et al.*, 1986; Schwer and Stunnenberg, 1988). The 7.5K promoter is transcribed in the early and late phase of infection from two different control regions: an upstream late ('TAAAT-type') and a downstream early promoter (Cochran *et al.*, 1985b). The I3 gene is also expressed in both stages of infection and transcription initiation occurs at an upstream control region used post-replication and a downstream early promoter (Schmitt and Stunnenberg, 1988). The I8 promoter transcribed post-replication is located ~300 bp upstream of the first AUG of the I8 open reading frame (this publication). Neither I3 nor I8 contains a TAAAT-motif. We regard and will refer to the I8 and the upstream I3 promoter as intermediate, based on the results described here.

A subset of post-replicative genes is expressed in the absence of DNA synthesis upon transfection into vaccinia-infected cells

Transfection of vaccinia-infected cells was carried out as described by Cochran *et al.* (1985a) and the cells were subsequently incubated in the presence of hydroxyurea, an inhibitor of DNA synthesis. Transcripts originating from either the viral (genomic) or transfected copy of the different promoters were monitored independently by S1 mapping (Figure 2). The vast majority of the transcripts originating from the transfected plasmid containing the I3 promoter fragment are initiated at the intermediate RNA start site (Figure 2B). In contrast, the intermediate start site of the genomic I3 promoter is not used (Figure 2A). A similar result is obtained with the I8 promoter, i.e. a transfected chimeric construct is transcribed, whereas the genomic promoter is inactive (Figure 2). Furthermore, transcripts from the transfected 11K and 7.5K late promoters are not detectable in the absence of DNA synthesis (Figure 2B). Transcripts from the transfected 7.5K early promoter construct could not be detected.

An intermediate I8-promoter chloramphenicol acetyltransferase (CAT) construct was expressed in the absence

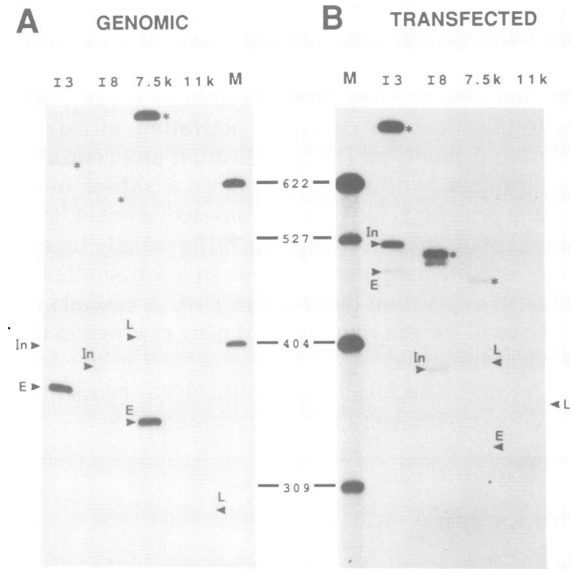

Fig. 2. S1 analysis of RNA derived from genomic and transfected promoters. Monolayers of HeLa cells were infected with w.t. virus (**A**) or infected with w.t. virus and transfected with plasmid DNA (**B**) and subsequently incubated for 9 h in the presence of 5 mM hydroxyurea. RNA was extracted and subjected to S1 analysis. (**A**) S1 protected fragments of transcripts originating from the genomic promoters of I3 (**lane 1**), I8 (**lane 2**), 7.5K (**lane 3**) and 11K (**lane 4**). (**B**) S1 protected fragments of transcripts originating from the transfected plasmids containing the I3 promoter (**lane 1**), I8 promoter (**lane 2**), 7.5K promoter (**lane 3**) and 11K promoter (**lane 4**). M, Labelled *Hpa*II-digested pBR322 DNA size markers. The arrowheads indicate the positions of the DNA fragments which would be protected by transcripts derived from the early, intermediate and late start sites. The asterisks indicate the position of the input probe.

of DNA replication if transfected into cells infected with wild-type (w.t.) virus (Figure 3). Accumulation of CAT activity was observed in cells infected with w.t. virus and transfected with an 11K−CAT chimeric construct, whereas background levels were obtained in the presence of hydroxyurea (Figure 3, HU). This result is in agreement with the results obtained with the transfected 28K late promoter

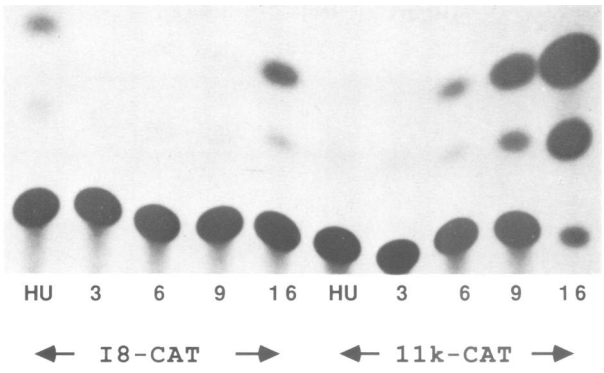

Fig. 3. CAT activities. Lysates were prepared from HeLa cells infected with w.t. virus and transfected with plasmids containing the I8−CAT and the 11K−CAT chimeric constructs respectively, harvested at different times after infection (as indicated in hours). HU, Incubated in the presence of 5 mM hydroxyurea and harvested 9 h post-infection.

(TAAAT-type) fused to the CAT gene in the presence of cytosine arabinoside (Weir and Moss, 1987).

It can be concluded that transfected intermediate I3 and I8 genes are transcribed in the absence of DNA replication, whereas the genes within the viral genome are not transcribed. In contrast, transfected 11K and 7.5K ('TAAAT'-type) promoters cannot be activated prior to DNA replication.

DNA replication is essential for derepression of intermediate transcription but not sufficient for late transcription

Hydroxyurea inhibition of the enzyme ribonucleotide reductase, which is involved in DNA replication, is known to be reversible in several eukaryotic tissues (Thelander and Reichard, 1979). We anticipated that this might also be the case with the reductase enzyme encoded in the viral genome (Slabaugh and Mathews, 1986). Monolayers of HeLa cells were infected with wild-type virus and transfected with a plasmid containing a chimeric construct consisting of the 11K late promoter and the dehydrofolate reductase (dhfr) gene. The cells were incubated in the presence of hydroxyurea, and hydroxyurea was removed to allow DNA replication to begin. Simultaneously, cycloheximide was added to inhibit *de novo* protein synthesis. The different intermediate and late transcripts were mapped simultaneously. In the presence of cycloheximide, RNA transcripts initiated at the intermediate start site of the viral I3 gene are readily detectable by S1 mapping (Figure 4, lane 1). The ratio between intermediate and early I3 transcripts obtained in the presence of cycloheximide is comparable with that obtained without cycloheximide treatment (Schmitt and Stunnenberg, 1988). We conclude that DNA replication is essential and sufficient for activation of intermediate genes after release of the hydroxyurea block and that *de novo* protein synthesis is not required after the onset of replication.

In contrast, we cannot detect transcripts from either the viral 11K gene or from the transfected 11K−dhfr construct in the presence of cycloheximide (Figure 4, lane 1). Transcripts from the 11K promoter are only generated after release of the cycloheximide block (Figure 4, lane 2). This result shows that DNA replication *per se* is not sufficient for activation of the late (TAAAT-like) genes; *de novo* synthesis of (intermediate) proteins is also essential.

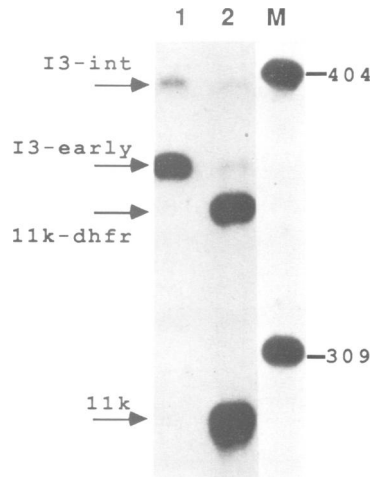

Fig. 4. S1 analysis of transcripts from the I3 and 11K promoters which were mapped simultaneously. Monolayers of HeLa cells were infected with the w.t. virus and transfected with the plasmid p32−dhfr (Hänggi *et al.*, 1986). **Lane 1**, incubated for 5 h with hydroxyurea added immediately after transfection and subsequently for 4 h with cycloheximide in the absence of hydroxyurea. **Lane 2**, as before with an additional 10 h in the absence of inhibitors. RNA was extracted and subjected to S1 analysis. The arrows indicate the DNA fragments protected from nuclease digestion by transcripts originating from genomic I3 and 11K transcripts and transcripts from the transfected plasmid p32−dhfr (11K−dhfr).

Temporal expression of polypeptides synthesized post-replication

Hydroxyurea treatment synchronizes the infection at the onset of replication and the temporal order of expression of post-replicative proteins can be determined by release of the inhibitory block. For this purpose, cells were either infected or mock-infected and incubated first with hydroxyurea and subsequently with cycloheximide to enrich for intermediate mRNA. Polypeptides were pulse labelled with [^{35}S]methionine in the presence of hydroxyurea, and at different time-points after cycloheximide release and analysed on a SDS−polyacrylamide gel (Figure 5). The pattern of labelled polypeptides synthesized in mock-infected cells (Figure 5, lanes 1 and 2), in the presence of hydroxyurea (lanes 2 and 3), and after release of the cycloheximide block (lanes 1 and 4−8) are clearly different, reflecting viral infection and shut-off of host mRNA translation. Note, that a shut-off of host protein synthesis is not evident prior to DNA replication (cf. lanes 2 and 3). We have indicated some of the polypeptides which are expressed immediately after cycloheximide release and their synthesis decreases after 1−2 h (closed arrowheads). This pattern of expression is anticipated for an exclusively intermediate gene product (such as I8). The pattern of expression of late gene products, exemplified by 4b and 28K which are of the TAAAT-type (Rosel and Moss, 1985; Weir and Moss, 1987) reveals a short lag period and maximal expression after 1−2 h (indicated by arrows). This delayed expression is in agreement with the observation that late transcription depends on *de novo* protein synthesis post-replication.

In vitro transcription

Run-off transcripts correctly initiated at vaccinia promoters are not detectable in extracts from mock-infected cells, demonstrating that transcription is dependent on viral

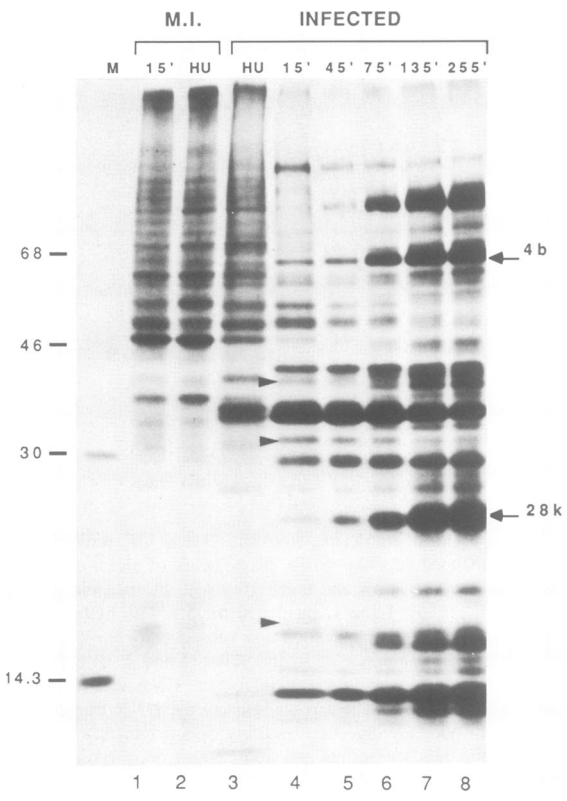

Fig. 5. Pulse labelling of polypeptides. Monolayers of HeLa cells were either mock-infected (M.I.) or infected with w.t. virus (**lanes 3–8**) and incubated in the presence of [^{35}S]methionine for 15 min after a methionine starvation of 15 min. Cells were either incubated for 16 h with hydroxyurea (**lanes 2** and **3**) or first for 6 h with hydroxyurea and subsequently for 10 h with cycloheximide and labelled after cycloheximide release at the time indicated (**lanes 1** and **4–8**). Polypeptides were applied to a SDS–polyacrylamide gradient gel (10–15%). M, ^{14}C-labelled high mol. wt protein markers. Typical late proteins are indicated by arrows and intermediate proteins are marked by arrowheads.

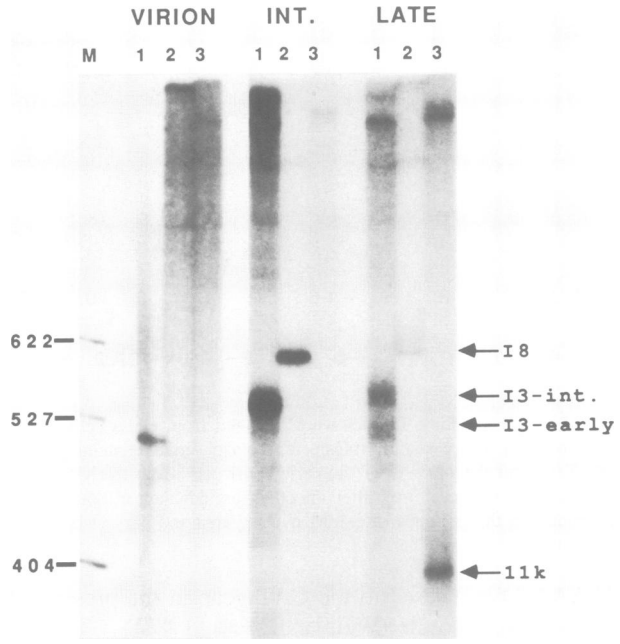

Fig. 6. *In vitro* transcription. Run-off transcription assays using an extract from purified virions, a whole cell extract prepared from HeLa S3 cells infected with w.t. virus harvested 14 h.p.i. (late) or from HeLa S3 cells infected with w.t. virus and incubated in the presence of 5 mM hydroxyurea (Int.). Truncated plasmids carrying the I3, I8 and 11K promoters (**lanes 1, 2** and **3** respectively) were used as templates. The arrows indicate the position of the RNA transcripts as expected on the basis of RNA start site and truncation. M, Labelled *Hpa*II-digested pBR322 DNA size markers.

proteins. This property may allow analysis of regulatory control mechanisms *in vitro* such as replication-dependent activation. Run-off transcription assays using cell-free extracts from purified virus particles result in transcripts initiated at vaccinia early promoters (Rohrmann and Moss, 1985; Golini and Kates, 1985). Transcription initiation in whole-cell extracts derived from infected HeLa cells harvested 14 h post-infection (h.p.i.) occurs at both late and early vaccinia promoters (Schwer and Stunnenberg, 1988). The latter activity might emanate from co-extracted assembled virions present within the infected cells at this stage of infection (14 h.p.i.).

The transfection experiments indicated that enzymes and *trans*-acting factors necessary for intermediate transcription are present in virus-infected cells before onset of DNA replication. Therefore, whole-cell extracts were prepared from virus-infected HeLa cells incubated for 9 h in the presence of hydroxyurea, referred to as intermediate extracts. The specificity of transcription was compared with a virion extract and a whole cell extract derived from virus-infected cells harvested 14 h.p.i., called late extract. Run-off transcripts initiated at the early start site of I3 are generated in virion extracts, whereas intermediate and late promoters are not recognized (Figure 6). Run-off transcription assays, using intermediate extracts, result almost exclusively in transcripts initiated at the I3 intermediate (I3-int) and I8 start sites. S1 mapping confirmed that the sites of transcription initiation *in vitro* and *in vivo* at the I8 promoter (Figure 7) as well as at the I3-int promoter (data not shown) are identical. A very low level of early transcription in hydroxyurea extracts can be detected by S1 mapping (data not shown). Run-off assays using late extracts give rise to late transcripts (Figure 6) having a 5′ poly(A) head as previously described (Schwer and Stunnenberg, 1988). Transcripts initiated at early and intermediate start sites are also generated.

We conclude that the pattern of transcription of the intermediate genes obtained in our *in vitro* extracts reflects the temporal order of expression of these genes observed *in vivo*. The fact that late transcripts are generated in late extracts, but not in intermediate extracts, confirms that (intermediate) proteins are necessary for late transcription.

Discussion

Vaccinia virus genes are classified as early and late, depending on whether they are expressed prior to or after the onset of DNA replication. Our results demonstrate that the genes expressed post-replication have to be divided into two classes, intermediate and late. The intermediate genes are expressed immediately post-replication and are required for onset of late gene expression. The late promoters are characterized by the presence of a conserved and essential TAAAT-sequence motif at the site of transcription initiation (Hänggi *et al.*, 1986; Schwer and Stunnenberg, 1988).

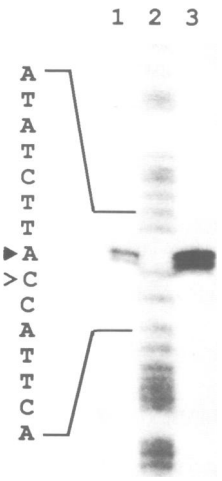

Fig. 7. S1 analysis of *in vitro* and *in vivo* I8 transcripts. RNA extracted from HeLa S3 cells infected with w.t. virus and incubated in the presence of hydroxyurea and cycloheximide subsequently (**lane 1**). Purine-specific cleavage of S1 DNA probe as described by Maxam and Gilbert (1980) (**lane 2**). RNA synthesized *in vitro* in a standard transcription assay using a whole cell extract from HeLa S3 cells infected with w.t. virus and incubated in the presence of hydroxyurea. A plasmid containing sequences from −255 to +395 relative to the I8 RNA start site was used as a template (**lane 3**).

Primer extension and S1 mapping experiments using *in vivo* and *in vitro* RNA indicate that the intermediate transcripts are transcribed in a co-linear fashion and do not acquire a poly(A) head (data not shown). In addition intermediate transcripts are not terminated *in vitro* in response to an early termination signal (data not shown). The intermediate I8 and I3-int promoters lack the TAAAT motif at the RNA start site. The similarity between the I3-int and I8 promoter in primary sequence and the distance of this motif to the RNA start site is striking (underlined sequences in Figure 1). Although this homology is only based on the comparison of two intermediate promoters, vaccinia promoters do not share long conserved sequence motifs. A detailed mutational analysis of the intermediate promoter is presently being undertaken to determine *cis*-acting promoter sequences.

Regulation of intermediate transcription

We have shown that transfected copies of intermediate genes can be expressed in virus-infected cells independent of DNA replication indicating that positive *trans*-acting factors required for intermediate transcription are present prior to the onset of DNA replication (Figures 2 and 3). This is in agreement with the observation that intermediate transcripts are generated in a cell-free extract prepared from vaccinia-infected cells incubated in the presence of hydroxyurea (Figure 6). In principle, the viral genome is accessible to *trans*-acting factors at this stage of infection, since a T7 promoter−β-galactosidase construct inserted into the vaccinia tk locus is transcribed by T7 RNA polymerase in the absence of DNA replication (Fuerst *et al.*, 1987). Nevertheless, the intermediate genes within the viral genome are not activated prior to DNA replication. An explanation is that the interaction of positive *trans*-acting factors with the intermediate regulatory sequences is prevented by a specific repressor. It is tempting to speculate that the extensive sequence homology between the I8 and I3-int promoters represents the binding site of both positive and negative transcription factor(s) and that the binding is mutually exclusive.

DNA replication *in cis* is essential for derepression of intermediate transcription *in vivo*. It has been reported that DNA replication can erase the 5S RNA transcription complex *in vitro* (Wolffe and Brown, 1986). In contrast, transcription through the 5S promoter by RNA polymerase III does not dislodge the committed transcription complex (Bodenhagen *et al.*, 1982). The genomic organization at the vaccinia I3 locus is such that transcription initiated at the early I4 RNA start site proceeds through the intermediate I3 promoter and terminates ~25 bp downstream of the intermediate I3 RNA start site as schematically indicated in Figure 1 (Schmitt and Stunnenberg, 1988). This implies that maximally 8 bp between the termination site of I4 and the early RNA start site of I3 are not transcribed in the early phase of infection. Therefore, DNA replication but not early transcription can derepress intermediate transcription.

Cascade model

The discovery of an intermediate class of genes necessitates a revision of the model for vaccinia virus reproduction and indicates a more elaborate control of gene expression during the viral infectious cycle. Early transcription starts immediately upon entry of the virus into the cell and does not require *de novo* synthesis of viral factors or enzymes; all necessary components are present in the infectious particle. The activation of intermediate transcription requires positive *trans*-acting factors which are present in an active state in the infected cells prior to the onset of DNA synthesis. The most obvious explanation is that these factors are encoded by early viral genes. Alternatively, host proteins or viral structural proteins might be involved in the activation of intermediate transcription as is the case for the structural protein Vmw65 in HSV transcription (O'Hare and Goding, 1988; Preston *et al.*, 1988). Whatever the origin of these factors, the presence of *trans*-acting factors is essential but not sufficient for the activation of intermediate transcription. Our results indicate that the intermediate genes are actively repressed and that viral DNA replication is required for derepression. Replication is not sufficient for the activation of late transcription; in addition *de novo* protein synthesis is essential. *Trans*-acting factors required for late transcription are (at least in part) encoded by intermediate genes. This is consistent with the observation that late transcripts are not detectable in intermediate extracts. It remains an open question whether activation of late transcription is solely dependent on the presence of intermediate *trans*-acting factors or whether DNA replication is also an essential step in this process. Finally, it seems likely that the factors involved in the regulation of transcription in the early phase of infection are late polypeptides, which are packaged into newly assembled virus particles and exert their action upon re-infection. The putative repressor of intermediate transcription could be such a late protein. The picture emerging for the control of gene expression during vaccinia virus infection closely resembles regulatory mechanisms involved in developmental control in eukaryotes and during infections with nuclear viruses (e.g. HSV, SV40 and Ad) despite the fact that vaccinia is a cytoplasmic virus.

Our cell-free extracts transcribe early, intermediate and late genes in a way which reflects the temporal expression

of these genes *in vivo*. It should now be possible by fractionation/complementation to characterize and purify positive and negative *trans*-acting factors involved in the differential regulation of vaccinia virus gene expression.

Materials and methods

Virus and cells

Vaccinia virus (strain WR) was grown in HeLa S3 suspension cultures that were maintained in Eagle's medium containing 5% new-born calf serum. HeLa monolayers were maintained in DMEM supplemented with 10% fetal calf serum.

Construction of plasmids

The I3 promoter fragment used for transient and *in vitro* transcription assays comprises sequences from −256 (*Pst*I site) to +168 (*Cla*I site) relative to the intermediate RNA start site. The I8 promoter fragment used for transient assays comprises sequences from −120 (*Cla*I site) to +26 (*Hpa*I site) relative to the RNA start site. The 7.5K promoter fragment used for transient expression comprises sequences from −132 to +31 relative to the early RNA start site. The 11K promoter fragments, used in transient expression and *in vitro* transcription experiments, comprise sequences from −32 to +8 relative to the RNA start site (Hänggi *et al.*, 1986).

Transient expression

Subconfluent HeLa cell monolayers were infected with wild-type virus in phosphate-buffered saline (m.o.i. of 30). After 30′ at RT, medium with 5 mM hydroxyurea was added. Ten micrograms of calcium-phosphate-precipitated plasmid DNA were added per 1.5×10^6 cells. RNA was extracted 9−12 h post-transfection with guanidinium hydrochloride followed by CsCl purification as described by Maniatis *et al.* (1982).

Pulse labelling of polypeptides

HeLa cell monolayers were either mock-infected or infected with wild-type virus (m.o.i. of 30) and incubated in the presence of inhibitors as indicated in the figure legends. Polypeptides were labelled for 15 min with 30 μCi/ml of L-[^{35}S]methionine after an incubation of 15 min in methionine-free medium. Cells were harvested with 0.02 N NaOH, 0.5% SDS and one volume of 2× Laemmli buffer (Laemmli, 1970) was added. The samples were boiled for 10 min and applied to a 10−15% polyacrylamide−SDS gradient gel (Laemmli, 1970).

S1 mapping

Total RNA (5−10 μg) was hybridized overnight at 44°C to asymmetrically end-labelled DNA probes (Maniatis *et al.*, 1982). S1 nuclease digestion was for 1 h at RT with 500 U/ml. Protected fragments were analysed on a sequencing gel.

CAT assays

HeLa cell monolayers were infected, transfected and incubated for the indicated times in the presence or absence of 5 mM hydroxyurea. Cells were harvested at the various times and CAT activities were measured as described by Gorman *et al.* (1982).

Preparation of transcription extract

Whole cell extracts from infected HeLa suspension cultures were prepared according to Schwer and Stunnenberg (1988). Late extracts were prepared from infected cells 14 h post-infection, whereas intermediate extracts were prepared from infected cells incubated in the presence of 5 mM hydroxyurea for 9 h. Virion extracts were prepared as described by Rohrmann and Moss (1985).

Transcription conditions

Transcription assays were performed at 30°C for 45 min in a final volume of 10 μl. Whole cell extracts: 50% (v/v) extract, 25 mM Hepes (pH 7.9), 50 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 8.5% glycerol, 0.5 mM of GTP, CTP, and ATP and 0.05 mM of UTP, 10 μCi of [α-^{32}P]UTP (800 Ci/mmol) and 100 μg/ml of linearized plasmid DNA. Virion extracts: 10% (v/v) extract, 40 mM Hepes (pH 7.9), 10 mM Tris (pH 8), 25 mM KCl, 0.02 mM EDTA, 5 mM DTT, 1.5% glycerol, 0.5 mM of GTP, ATP, and ATP, and 0.05 mM of UTP, 5 μCi of [α-^{32}P]UTP (800 Ci/mmol) and 50 μg/ml of linearized plasmid DNA.

Sources of materials

Restriction enzymes and hydroxyurea were purchased from Boehringer, S1 nuclease from Pharmacia, radioactive nucleotides and ^{14}C-labelled mol. wt protein markers from Amersham, and media for cell culture from Gibco.

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