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Mutational Analysis of a Vaccinia Virus Intermediate Promoter In Vivo and In Vitro

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The expression of the vaccinia virus intermediate I3 gene depends on *trans*-acting factors which are present in an active state prior to DNA replication. However, activation of transcription requires DNA replication in *cis* (J. C. Vos and H. G. Stunnenberg, EMBO J., 7:3487–3492, 1988). We have made deletion and linker scanner mutations of the I3 promoter to determine the sequence requirements for transcriptional activity and the dependence of DNA replication. The I3 promoter appears to consist of two elements which are essential and sufficient for accurate transcription initiation both in vivo and in vitro. An upstream and a downstream sequence element were defined ranging from −20 to −9 and +1 to +9, respectively. The upstream element appears to be highly homologous to a sequence in the intermediate I8 promoter. A 3-bp substitution in the upstream I3 promoter element resulted in a change of transcriptional specificity from intermediate to late. Finally, the mutations did not result in an activation of the intermediate promoter prior to DNA replication.

The genes of vaccinia virus (for a review, see reference 15) can be classified into three groups (early, intermediate, and late) which are regulated in a temporal fashion (26). Early transcription starts immediately upon infection within the viral core which contains the entire early transcriptional apparatus. Synthesis of early viral polypeptides, uncoating of the virion, and subsequent DNA replication are required for activation of the postreplicative genes. Intermediate genes are transcribed immediately after replication even in the absence of continued protein synthesis. In contrast, the onset of late transcription is dependent on post-DNA-replication protein synthesis. These and other observations have led to the proposal of a cascade model, in which early proteins are required for intermediate transcription and protein(s) encoded by an intermediate gene(s) are required for the activation of late transcription (26). Finally, it has been shown that an early specific transcription factor (vaccinia early transcription factor [VETF]) is synthesized in the late phase of infection and packaged into the virion (2, 3).

Early transcription is regulated at the DNA level by two distinct sequence elements: a promoter and a terminator. Early promoters are distinguished by a sequence which extends from positions −28 to −13 bp relative to the start site of transcription (4). The early promoter-binding factor (VETF) recognizes this sequence and is analogous to the eucaryotic TATA box-binding protein in being essential for transcription initiation and in positioning of the start site of transcription (1, 3, 29). The sequence UUUUUNU in the nascent RNA chain is required for termination of early transcription. The capping enzyme complex has been identified as a termination factor (21).

Late promoters are characterized by a highly conserved TAAAT sequence at the start site of transcription (8). The initiation complex is thought to slip in a 3′-to-5′ direction at the three transcribed T residues within this motif, thereby generating a 5′ poly(A) head of relatively discrete size (19, 20, 22). Mutations within or downstream of the TAAAT motif may affect the length of the poly(A) head (5, 6). A late promoter element with a low sequence constraint, which is required for transcriptional activity, is located upstream of the RNA start site motif (5).

Intermediate promoters appear to play a pivotal role in the control of the viral life cycle as their activation depends upon DNA replication. The *trans*-acting factors necessary for the activation of intermediate gene transcription appear to be present prior to DNA replication as the product of early viral protein synthesis. This conclusion is based on the following observations. First, a transfected intermediate gene but not its genomic copy is transcribed prior to DNA replication in the presence of hydroxyurea, an inhibitor of DNA synthesis (26). Second, an intermediate specific cell extract can be derived from cells infected with vaccinia virus and incubated in the presence of hydroxyurea (26).

Replication as a means to regulate gene expression is a commonly used mechanism among DNA viruses (23). To explain the dependence of transcriptional activation on DNA replication, one can envision a conformational and/or biochemical change (e.g., methylation) of the template upon replication, an enhancement of transcription by factors involved in replication, or a clearance of the template from inhibitory proteins. Inhibition could be the consequence of binding of either a highly sequence specific repressor molecule, such as the lambda repressor (17), or DNA-binding proteins which bind tightly to DNA without a specific DNA sequence requirement, such as histones.

The intermediate I3 promoter was characterized by deletion analysis and linker scanner mutations. Transcription from the chimeric mutant promoter-reporter gene constructs were tested both in vivo and in vitro. Two sequence elements which are essential and sufficient for transcription were identified. Neither of the promoter mutants analyzed in this study circumvented the need for DNA replication as an essential step towards the activation of intermediate transcription of vaccinia virus.

MATERIALS AND METHODS

Virus and cells. Vaccinia virus (strain WR) and recombinants were grown in RK-13 or human 143 monolayers that were maintained in Eagle medium containing 10% fetal calf

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serum and were complemented with nonessential amino acids (143 cells). HeLa cell suspension cultures were maintained in Joklik medium.

Preparation of recombinant viruses. Recombinant viruses were prepared with the temperature-sensitive mutant ts7 (7) as a helper virus essentially according to the procedure of Kieny et al. (11).

RNA extraction. RNA from cells infected with vaccinia virus (multiplicity of infection of 5) was extracted 9 h postinfection with guanidinium hydrochloride as described previously (14). When indicated, infected cells were incubated in the presence of 10 mM hydroxyurea or 100 μg of cycloheximide per ml. RNA synthesized in in vitro transcription assays was extracted as described elsewhere (19).

S1 mapping. Total RNA (10 μg) extracted from cells infected with virus or RNA obtained from a standard in vitro transcription assay (20 μl, final volume) was hybridized overnight at 44°C to asymmetrically end-labeled DNA probes (14). S1 nuclease (Pharmacia) digestion was performed for 1 h at room temperature with 500 U/ml. Protected fragments were analyzed on sequencing gels.

In vitro transcription. Whole-cell extracts specific for intermediate transcription were prepared from HeLa S3 suspension cells infected with wild-type virus (multiplicity of infection of 5) and incubated in the presence of 10 mM hydroxyurea (19, 26). Transcription conditions were as previously described (26).

Construction of plasmids. An *Rsa*I restriction fragment (−36 to +44 with respect to the intermediate RNA start site) from the vaccinia virus *Hin*dIII I fragment carrying the I3 promoter (18, 26) was inserted into the *Sph*I restriction site of pUC18 (25). The *Sph*I restriction site was made blunt with T4 DNA polymerase. BAl 31 deletions were made using 0.1 U of the enzyme at room temperature in the buffer indicated by the manufacturer (Biolabs) and 5 μg of linearized plasmid (*Pst*I and *Hin*dIII for the 5′ and 3′ deletions, respectively). The reaction was terminated by phenol extraction, and ends were filled in with the large Klenow fragment of *Escherichia coli* DNA polymerase. The mutated I3 promoter fragments were subsequently inserted upstream of the chloramphenicol acetyltransferase (CAT) gene within pBluescript II KS$^+$ (Stratagene). The promoter mutant-CAT chimerae were inserted between the *Cla*I and *Eco*RI restriction sites of the thymidine kinase (tk) gene of vaccinia virus contained within pBluescript II KS$^+$.

Deletion endpoints were determined by sequencing. The flanking sequences and the junctions of the 5′-deletion mutations are as follows: Δ−30, ATCGATACCGTCGATG CAGT; Δ−24, ATCGATACCGTCGAGGTTAA; and, Δ−16, ATCGATACCGTCGAAAAAAC. Flanking sequences and junctions of the 3′-deletion mutations are as follows: Δ+36, TTATATTAAGCTT; Δ+30, ATATCATAAGCTT; Δ+17, AAAGTGTAAGCTT; and Δ+9, AATGAGTAAGCTT. Underlined sequences mark I3 promoter sequences. The *Hin*dIII restriction site at the downstream border of the 5′- as well as 3′-deletion mutations corresponds to the respective site at the beginning of the CAT gene.

Linker scanner mutations. Oligonucleotides containing the respective mutations flanked on each side by 12 bp of sequences complementary to the I3 promoter fragment were synthesized. Single-stranded templates were obtained from Bluescript-based plasmids by superinfection with phage. Synthesis of the second strand with the oligonucleotides as a primer and selection of the mutation were performed with the Amersham kit (RPN.1523) for site-directed mutagenesis.

RESULTS

BAL 31 deletions. The I3 gene is controlled by a tandem promoter, directing the synthesis of early and intermediate transcripts (18, 26). The intermediate start site of transcription is located 37 bp upstream of the early RNA start site. Initial experiments indicated that both the intermediate and early promoters are contained within a fragment extending from −36 to +44 bp relative to the intermediate RNA start site. This region, an *Rsa*I restriction fragment, was used to construct 5′ and 3′ BAL 31 deletions and cloned in front of the bacterial gene coding for the CAT enzyme. Chimeric I3 promoter-CAT genes were inserted into the tk locus of vaccinia virus by homologous recombination (13, 16). In this configuration, the translocated I3-CAT gene is transcribed in the same direction as the endogenous tk gene. Early transcripts from the tk promoter will be terminated downstream of the I3 intermediate start site of transcription initiation in response to the termination signal TTTTTAT present within the translocated I3 promoter fragment (18).

Nuclease S1 analysis was performed on the transcripts from the different promoter mutants. RNA was prepared from cells infected with recombinant virus and incubated in the presence (transcription before replication) or absence (transcription after replication) of hydroxyurea. RNA transcripts originating from the natural I3 promoter were mapped simultaneously and used as an internal standard in the S1 analysis. A schematic presentation of the S1 mapping is indicated in Fig. 1. The relative promoter strength was deduced from the ratio of intermediate to early transcripts derived from the natural I3 gene (internal control) compared with that of the chimeric translocated I3 promoter mutant-CAT constructs. The ratio of intermediate to early transcripts fluctuated from one experiment to the other because of differences in the multiplicities of infection used. However, within one RNA preparation, the ratio of early to intermediate transcripts was constant when transcripts from the natural I3 gene were compared with those from the translocated wild-type I3 promoter-CAT construct. A promoter mutation resulting in a decrease or increase of the promoter activity of either the early or the intermediate mutated promoter was reflected by a change in the ratio of early to intermediate transcripts compared with the transcripts from the natural I3 gene.

The parental I3 promoter fragment (−36 to +44) fused to the CAT gene and translocated into the tk locus directs the synthesis of both early and intermediate CAT transcripts in a ratio similar to that obtained from the natural I3 promoter (Fig. 2, lane 1) (26). This translocated promoter is regarded as a wild type and was used as the template for further mutagenesis. Deletions of 5′ sequences up to positions −30 or −24 did not alter the promoter activity significantly (Fig. 2, lanes 2 and 3, respectively). The apparent increase in CAT transcripts resulted from a difference in specific activity of the S1 probes. A deletion to position −16 resulted in a considerable reduction in intermediate transcription (Fig. 2, lane 4). The ratio of intermediate to early CAT transcripts was reduced 5- to 10-fold compared with the ratio of transcripts from the natural I3 gene. This indicates that important *cis*-acting sequences between −24 and −16 were removed. A further deletion up to position −5 abolished intermediate specific transcription, whereas early transcription was not affected (data not shown).

The I3 promoter was further delineated by 3′ deletions. Intermediate I3 promoter activity was not affected by a deletion to position +17 (Fig. 3, lanes 1 to 6). A comparison

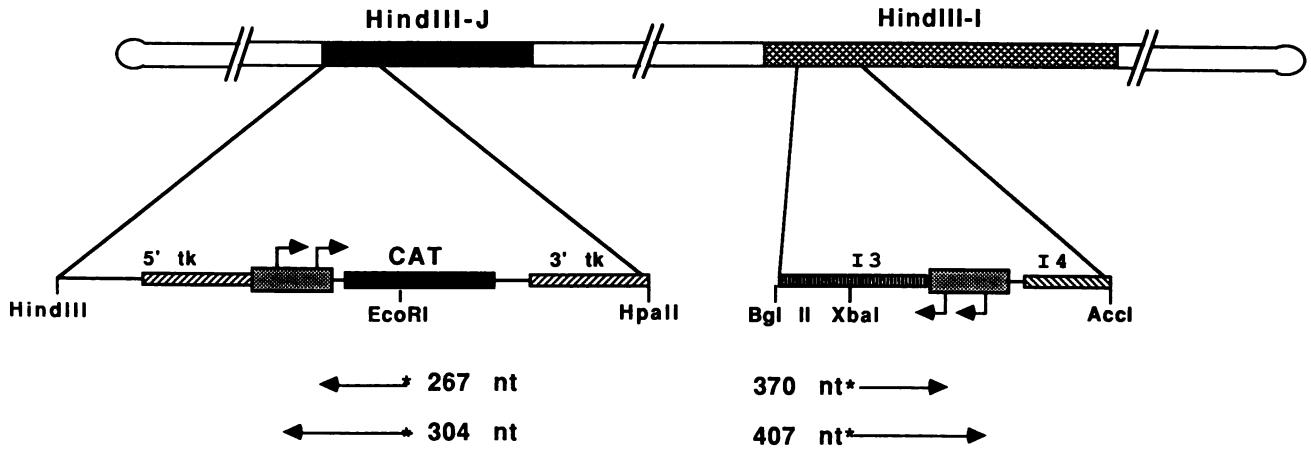

FIG. 1. Schematic representation of S1 mapping of transcripts from the natural and translocated I3 promoters. The locations of the I3-CAT constructs and the natural I3 gene within *Hin*dIII fragments J and I, respectively, are indicated. CAT transcripts are mapped from the *Eco*RI restriction site, and the natural I3 transcripts are mapped from the gene internal *Xba*I restriction site. The lengths of the protected fragments are indicated in nucleotides (nt).

between early and intermediate transcripts was not possible, since early transcription was reduced or abolished upon deletion of sequences upstream of position +30 (or −6 relative to the early start site) (Fig. 3, lanes 3 to 8). A 3′ deletion up to position +9 reduced the intermediate promoter activity (Fig. 3, lane 8), implying that sequences downstream of the intermediate RNA start site are important for intermediate promoter function. In contrast, DNA sequences downstream of the early RNA start site are not essential for early promoter activity, as their deletion has no effect (4; P. Hirschmann and H. G. Stunnenberg, unpublished observations). Mutations of sequences between the RNA start site and the binding site for the early promoter-binding factor (VETF) decreased the early promoter strength (Fig. 3, lanes 1 to 4). Mutant Δ+17 deleted part of the binding site of VETF and abolished early transcription (Fig. 3, lanes 5 and 6).

RNA was also prepared from cells infected with the recombinant viruses which were incubated in the presence of hydroxyurea. In this manner, only transcripts from promoters which are active prior to DNA replication can be obtained. Analysis of these transcripts should reveal whether any of the mutations within the intermediate promoter resulted in derepression prior to DNA synthesis, i.e., affected the recognition sequence of a presumed negative *trans*-acting factor. Neither the 5′- (data not shown) nor 3′-deletion mutations (Fig. 3, lanes 1, 3, 5, and 7) resulted in

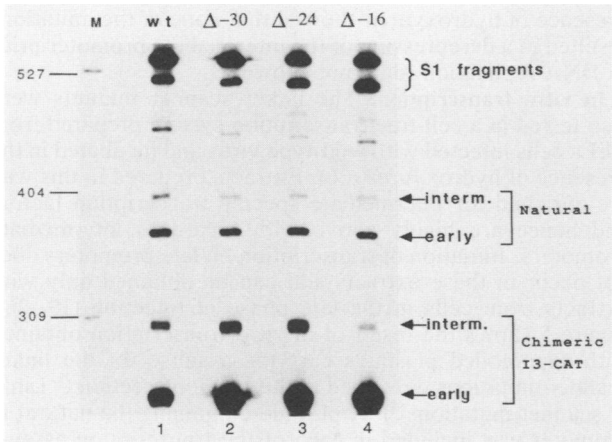

FIG. 2. S1 mapping of RNA prepared from cells infected with 5′-deletion recombinant viruses. RNA was prepared from cells infected with recombinant virus containing the I3 promoter fragment-CAT chimeric gene wt *Rsa*I fragment from −36 to +44, Δ−30, Δ−24, or Δ−16 at 9 h postinfection and subjected to S1 analysis. Arrows indicate the positions of the protected fragments of the early and intermediate (interm.) transcripts of the chimeric I3-CAT and natural I3 transcripts. The positions and the sizes (in base pairs) of ^{32}P-labeled, *Hpa*II-digested pBR322 size markers are shown (lane M).

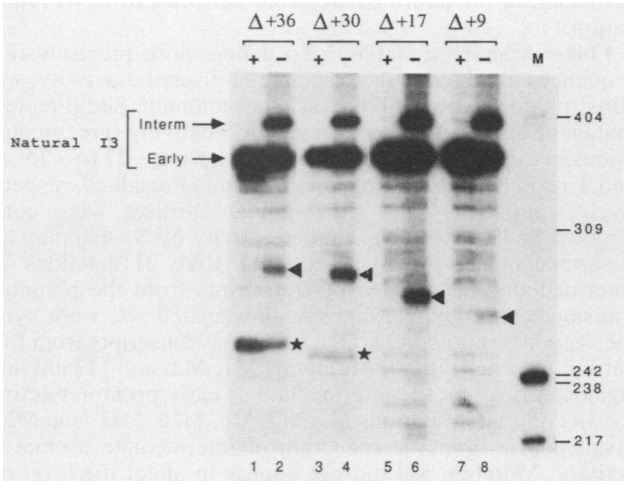

FIG. 3. S1 mapping of in vivo RNA prepared from cells infected with 3′-deletion recombinant viruses. RNA was prepared from cells infected with recombinant virus Δ+36, Δ+30, Δ+17, or Δ+9 at 9 h postinfection in the absence (lanes 2, 4, 6, and 8) or presence (lanes 1, 3, 5, and 7) of 10 mM hydroxyurea and analyzed by S1 mapping. Arrows indicate the positions of the protected fragments of the early and intermediate transcripts of the natural I3 transcripts. The expected sizes for the chimeric CAT-transcripts are marked by stars for early transcripts and by arrowheads for intermediate transcripts. The positions and sizes (in base pairs) of ^{32}P-labeled, *Hpa*II-digested pBR322 size markers are shown (lane M).

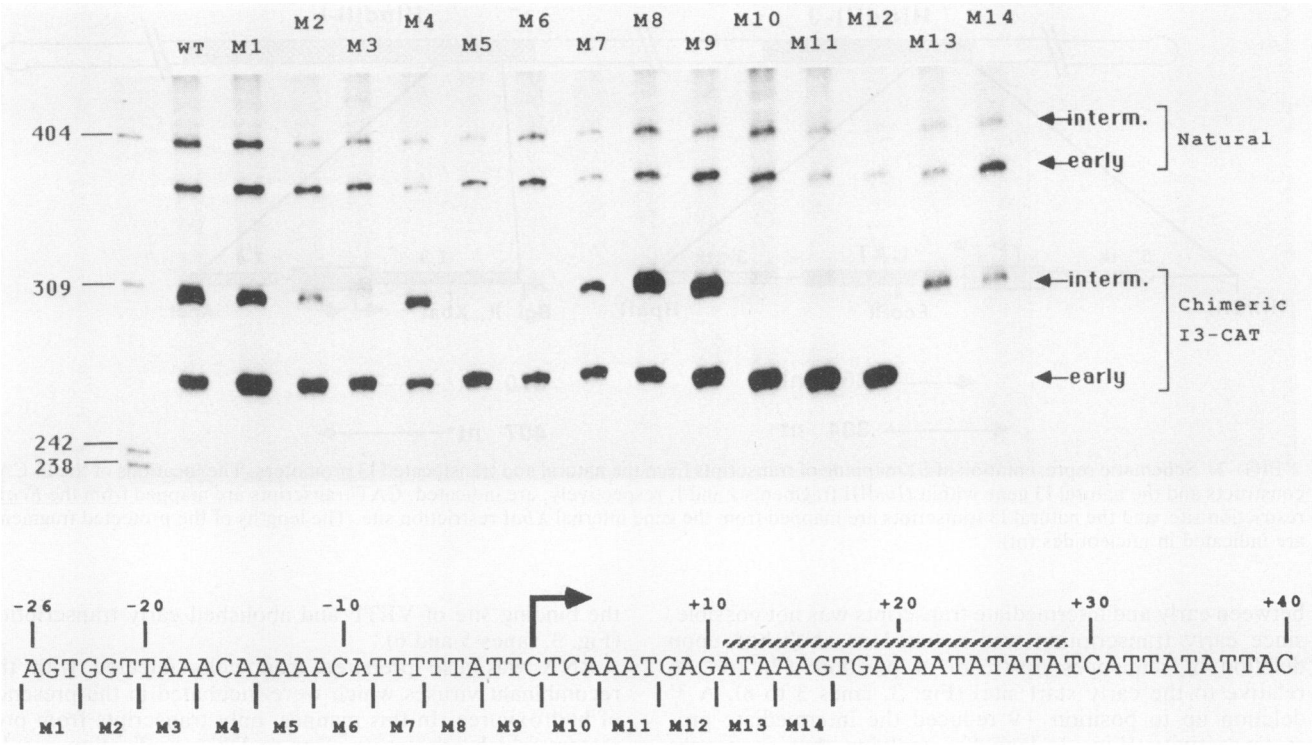

FIG. 4. S1 mapping of transcripts derived from the linker scanner mutants of the I3 promoter. Cells were infected with a recombinant virus containing a translocated I3 promoter with a 3-bp change (called M1 to M14, whereas the starting construct was designated wt). RNA was extracted 9 h postinfection and analyzed by S1 mapping. The chimeric I3-CAT transcripts were mapped simultaneously with the natural I3 transcripts. The expected protected fragments are indicated by arrows. The positions and the sizes (in base pairs) of ^{32}P-labeled, *Hpa*II-digested pBR322 size markers are shown (lane M). The sequence of the starting fragment and the positions of the mutations M1 to M14 are indicated below the lanes. The shaded box above the sequence indicates the binding site for the early factor VETF. The numbering is relative to the intermediate RNA start site which is indicated (arrow).

activation of the intermediate promoter prior to DNA replication.

Linker scanner mutations. To define more precisely the sequences involved in intermediate I3 promoter activity, we constructed a series of linker scanner mutants. Site-directed mutagenesis was used to change consecutively three nucleotides in the promoter region extending from −27 to +15. A and T residues were changed into C and G residues, respectively, and vice versa. Recombinant viruses were constructed and tested for promoter activity by S1 mapping as described in the previous section ("BAL 31 deletions"). Intermediate and early CAT transcripts from the parental translocated promoter fragment (designated wt) were synthesized in a ratio comparable to that of transcripts from the natural I3 gene (Fig. 4). Mutations M1, M2, and M7 did not significantly affect the intermediate or early promoter activity. In contrast, mutations M3, M5, M6, M10, M11, and M12 resulted in a dramatic reduction of intermediate promoter activity. Mutation M4 did not appear to affect the level of CAT transcripts; however, careful inspection suggested that the intermediate RNA start site was shifted to a more downstream position. Mutants M13 and M14 were active with respect to intermediate transcription, whereas early transcription was abolished. These mutations alter the binding site for the early promoter binding factor VETF (Fig. 4) (29). Mutations M8 and M9 resulted in an increase in intermediate promoter activity. These two mutations as well as mutation M7 removed the *cis*-acting sequence (U_5NU) required for termination of early transcription (30). Taken together, these data define two crucial sequence elements from −20 to −9 and +1 to +9. S1 mapping of RNA transcripts obtained from infected cells incubated in the presence of hydroxyurea showed that none of the mutations resulted in a derepression of the intermediate promoter prior to DNA replication (data not shown).

In vitro transcription. The linker scanner mutants were also tested in a cell-free transcription system prepared from HeLa cells infected with wild-type virus and incubated in the presence of hydroxyurea (26). Extracts prepared in this way are enriched for intermediate specific transcription factors and hence are highly active with regard to intermediate promoters. Initiation of transcription on late promoters does not occur in these extracts and can be obtained only with extracts from cells in the late phase of infection (19, 26). Figure 5 shows the result of in vitro transcription obtained with supercoiled plasmids carrying a subset of the linker scanner mutations described in the previous section ("Linker scanner mutations"). A plasmid containing the natural I3 promoter was included in each of the transcription assays, and the transcripts were analyzed by S1 mapping as described above. The wild type and the mutations M1, M2, M7, M8, M9, M13, and M14 did not affect the intermediate promoter activity in vitro significantly compared with activity of the natural I3 promoter (Fig. 5 and data not shown). Mutations M3, M6, and M12 resulted in a strong reduction in the level of transcription in vitro, whereas transcripts from the promoter mutations M4, M5, M10, and M11 could not be detected in vitro (Fig. 5 and data not shown). With the

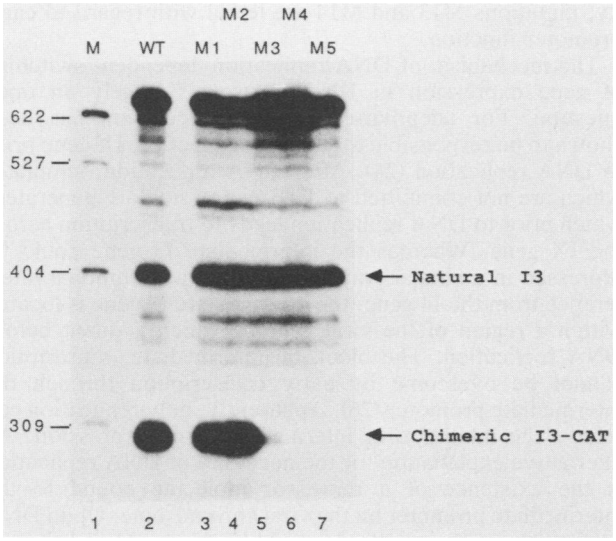

FIG. 5. S1 mapping of in vitro-synthesized RNA. An intermediate specific extract prepared from HeLa cells infected with vaccinia virus and incubated with 10mM hydroxyurea for 16 h was primed with a plasmid containing the natural I3 promoter and a second plasmid carrying a linker scanner mutation. The linker scanner mutations were wt (lane 2), M1 (lane 3), M2 (lane 4), M3 (lane 5), M4 (lane 6), and M5 (lane 7). RNA was prepared and subjected to S1 analysis. The S1-protected fragments corresponding to the intermediate transcripts are indicated by arrows. Lane M, ^{32}P-labeled, *Hpa*II-digested pBR322 size markers (sizes are indicated in base pairs on the left).

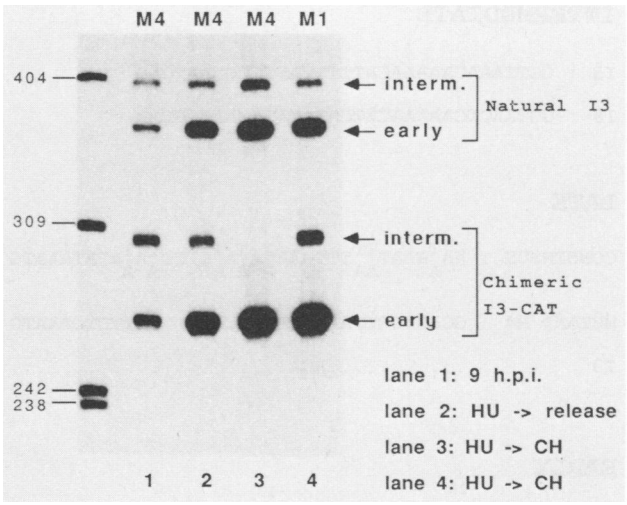

FIG. 6. Expression of M4 requires protein synthesis postreplication. RNA was prepared from cells infected (multiplicity of infection of 5) with recombinant virus M4 or M1 and incubated under different conditions: for 9 h in the absence of any inhibitors (lane 1); for 4 h in the presence of hydroxyurea (HU) and for an additional 5 h in the absence of drugs (lane 2); for 4 h in the presence of hydroxyurea and subsequently for 5 h in the presence of cycloheximide (CH) (lanes 3 and 4). The chimeric I3-CAT transcripts were mapped simultaneously with the natural I3 transcripts by using nuclease S1. The expected protected fragments are indicated by arrows. The positions and the sizes (in base pairs) of ^{32}P-labeled, *Hpa*II-digested pBR322 size markers are shown on the left. h.p.i., Hours postinfection.

exception of data from mutation M4, these results reflect the data obtained in vivo.

M4 is a late promoter. Mutant M4 did not appear to have intermediate promoter activity in vitro (Fig. 5, lane 6) but did show a wild-type level of expression in vivo (Fig. 4 and 6, lane 1). We therefore considered the possibility that the M4 mutation had converted this intermediate promoter into a late promoter. In order to test this hypothesis, recombinant viruses M1 and M4 were used to infect cells in the presence of hydroxyurea to block DNA replication. Upon substitution of hydroxyurea for cycloheximide, an inhibitor of protein synthesis, DNA replication is initiated and intermediate genes are derepressed (26). Early as well as intermediate transcripts originating from the promoter mutation M1 were readily detectable under these conditions (Fig. 6, lane 4). However, only early transcripts could be detected originating from mutation M4 (Fig. 6, lane 3). De novo protein synthesis after the onset of DNA replication was required in order to obtain transcripts from the upstream start site (Fig. 6, lane 2), which is characteristic of late gene expression (26). The S1-protected fragments obtained with the M1 and M4 transcripts were of similar lengths, in contrast to the results presented in Fig. 4. This could be due to small differences in the conditions of S1 digestion, which are critical when late transcripts are mapped with a 5′ poly(A) head (6, 19). We conclude that mutation M4 is a late promoter.

DISCUSSION

The intermediate I3 promoter was characterized by mutational analysis and shown to be contained within a small region of 30 bp which consists of an upstream element from −20 to −9 and a downstream element from +1 to +9 relative to the RNA start site. These elements are sufficient to direct intermediate transcription both in vivo and in vitro. The size and the position of the upstream element relative to the RNA start site are comparable to those of similar elements in early and late promoters of vaccinia virus (4, 5).

We pointed out previously (26) that the sequence in the upstream part of the intermediate I3 promoter is highly homologous to that of the I8 promoter, having 10 of 12 bases in common (see Fig. 7). The mutational analysis presented here reveals that this element is essential for intermediate promoter activity. The downstream elements of the two known intermediate promoters do not share an obvious sequence homology. Furthermore, the distance between the RNA start site and the upstream promoter element is not conserved. For early transcription, the selection of the RNA start site is determined by VETF in a manner comparable to the TATA-box factor of eucaryotic RNA polymerase II promoters (1, 3). For intermediate transcription, it is likely that an intermediate specific factor will bind to the upstream promoter element and direct the viral RNA polymerase to the promoter. Whereas the start site region of early transcription does not seem to require specific sequences, the intermediate and late promoters are highly sensitive to mutations at or downstream of the RNA start site. We are currently investigating whether this reflects the specific binding of either a *trans*-acting factor or RNA polymerase or the potential sequence constraints for the formation of an open complex. The latter might result in a greater variation in the positioning of the RNA start site, as observed with the I3 and I8 promoters. In support of this hypothesis, it has been shown for eucaryotic RNA polymerase I that a transcription initiation factor binds independently to an up-

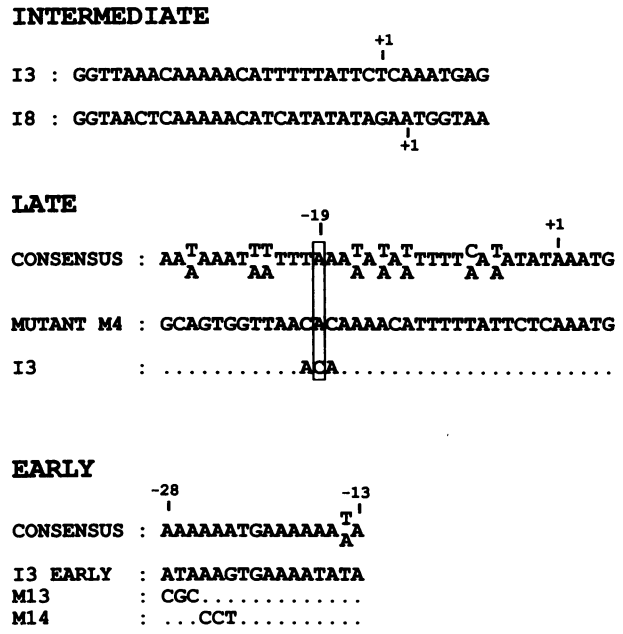

FIG. 7. Alignment of vaccinia virus promoter sequences of the three temporally regulated gene classes. The sequences of the intermediate I3 and I8 promoters (18) are compared. The early consensus sequence is aligned with the early part of the tandem I3 promoter and with promoter mutations M13 and M14. The late consensus sequence (5) is aligned with the intermediate I3 promoter and with mutation M4. Position −19, which is apparently critical for promoter specificity, is boxed. The numbering relates to the distance from the respective RNA start sites.

stream sequence element and by virtue of protein-protein interactions directs RNA polymerase to the RNA start site (12). The binding of RNA polymerase I is not influenced by the DNA sequence at or downstream of the RNA start site. However, transcription can be abolished by mutations within these sequences (12). Similarly, transcription in *E. coli* is a multistep process in which sequences in the transcribed region are involved in late steps of transcription. It was shown by Kammerer et al. (10) that a change within downstream sequences can affect promoter strength by more than 10-fold, although the recognition of the central core region by RNA polymerase is not altered.

The finding that mutation M4 converted the intermediate promoter into a late promoter was surprising. By sequence comparison with the late consensus sequence, a better match can be obtained with the natural promoter than with mutation M4 because the consensus consists solely of A · T base pairs. The C residue at position −19 in the natural promoter might be detrimental to late promoter activity. We cannot formally exclude the possibility that the natural I3 promoter or any of the functional mutants do intrinsically have a low level of late promoter activity. However, the relative promoter strength as determined in vitro in an intermediate specific cell extract is similar to those obtained in vivo, with the exception of mutation M4, which is shown to be a late promoter.

The early I3 promoter sequence is a very good match to the early consensus sequence, as postulated by Davison and Moss (4) (Fig. 7). The 5′ border of the early promoter as determined by these investigators is confirmed in this study. Whereas mutation M12 does not affect early promoter activity, mutations M13 and M14 are lethal with regard to early promoter function.

The mechanism of DNA replication-dependent switching of gene expression in DNA viruses is largely an open question. For adenovirus, promoter occlusion has been shown to be responsible for the silencing of the IX gene prior to DNA replication (24). After DNA replication, templates which are not committed to E1B expression are generated, which prior to DNA replication leads to transcription across the IX gene. Whereas the intermediate I3 gene could be repressed in a similar way because of transcriptional interference from the I4 gene, the intermediate I8 gene is located within a region of the viral genome which is silent before DNA replication. The block on intermediate transcription cannot be overcome by early transcription through the intermediate promoter (26). Apparently, only replication can relieve the inhibition of intermediate gene expression. An alternative explanation for the necessity of DNA replication is the existence of a repressor molecule bound to the intermediate promoter on the viral chromosome. Upon DNA replication, such a molecule could be displaced and titrated out by newly synthesized templates. From the presented mutational analysis, it can be deduced that if a sequence-specific repressor exists, it must contact the upstream and downstream promoter elements simultaneously, since no mutant was obtained, which obviates the need for DNA replication. To date, we have been unable to detect a sequence-specific repressor with the anticipated characteristics by gel retardation assays using either a virion extract or a cytoplasmic extract of cells infected with vaccinia virus. A further possibility is that a chromatinlike structure is adopted by the viral chromosome prior to DNA replication, thus preventing transcription. Several nonspecific DNA-binding proteins are known to be associated with the viral chromosome (e.g., the vaccinia virus VP8 protein [28]). In this respect, it can be envisioned that early transcription units are marked on the viral chromosome by the binding of VETF. By analogy, assembly of DNA into nucleosomes inhibits eucaryotic polymerase II transcription in vitro. Preincubation of the template with the TATA-box factor overcomes the inhibitory effect of nucleosome assembly (27). A totally different mode of replication-dependent switching could be a conformational change in the DNA template upon replication, a modification of the DNA, or an interplay of replication and transcription factors in vivo, as has been reported for late phage T4 promoters (9). We are currently addressing these questions.

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