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Evidence for Simian Virus 40 Late Transcriptional Control: Mixed Infections of Wild-Type Simian Virus 40 and a Late Leader Deletion Mutant Exhibit *trans* Effects on Late Viral RNA Synthesis

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Mixed infections involving equal multiplicities of wild-type simian virus 40 and viable deletion mutant *dl*861 resulted in decreased cytoplasmic levels of wild-type-derived late mRNA, as well as very low to undetectable levels of mutant-derived late mRNA, as compared with individual infections. The *dl*861 deletion removes 16 to 25 base pairs from the late leader region. This deletion was shown to be the direct cause of the mixed-infection effect; replacement of the deletion with wild-type sequences restored normal levels of late mRNAs in mixed infections. Other viral functions, e.g., early gene expression and replication, were found to be unaffected by the *dl*861 deletion. Further examination of the mixed-infection effect showed that the levels of unspliced nuclear precursors of late mRNA, derived from both the mutant and wild-type genomes, were decreased or undetectable, in accord with the cytoplasmic results. Thus, the effect appears to be occurring at the transcriptional level. These data demonstrate a *trans*-acting effect on late transcription, which is detectable due to the presence of the *dl*861 mutant in the mixed infection. This finding is indicative of a diffusible factor which exerts a control on simian virus 40 late gene expression at the transcriptional level. A model for positive control of simian virus 40 late gene expression is presented.

The transcriptional controls operative in expression of the simian virus 40 (SV40) late genes have remained enigmatic despite much study over several years. Initiation of late transcription appears to be linked to regulatory functions of large T-antigen (1, 2, 7). This is demonstrated by mutants which produce a temperature-sensitive large T-antigen (*tsA* mutants); when such mutants are grown continuously at the nonpermissive temperature, they not only fail to initiate viral DNA synthesis but also fail to fully express the late genes (2, 7). Shifting *tsA* mutant-infected cultures to the nonpermissive temperature after the initiation of late transcription shows that the continuation of high levels of late transcription may be dependent on the permissive host cell line (1). This has been interpreted as indicating that for late transcription to occur, an interaction between large T-antigen and a cellular factor(s) must take place; this interaction may or may not be effected by the *tsA* mutation, depending on the particular cell line (1). Recently, Laub et al. (12) suggested that another level of late transcriptional control may be operating by an attenuation mechanism. Overall, these data indicate that the transcriptional switch from early to late gene expression may be quite complicated, possibly involving well-coordinated interactions between viral and host factors. In this communication, data are presented which suggest that a diffusible viral factor, other than large T-antigen, exerts transcriptional control on late viral gene expression. The deletion mutant which allowed the study of this phenomenon is within the late leader region and removes the translational start codon for the agnoprotein (10). Thus, the agnoprotein may be the putative regulatory factor.

MATERIALS AND METHODS

Cells and viruses. SV40 strain 776 and deletion mutants *dl*861 (4, 6), *dl*1263 (5) and *dl*1263A were grown in primary African green monkey kidney cells (AGMK cells; Flow Laboratories, Inc., Rockville, Md.) or the established AGMK line CV-1P. Cells were grown in Dulbecco minimal essential medium containing 10% fetal bovine serum. When confluent, the cells were infected with viruses in a small volume of Dulbecco medium containing 2% serum (4 ml per 150-cm^2 bottle). After adsorption for 1.5 h with rocking at room temperature, the cells were fed with Dulbecco medium

containing 2% serum and incubated at 37°C for appropriate times.

Preparation of specific hybridization probes of ^{32}P-labeled SV40 DNA. SV40 DNA was labeled in vivo as described previously (11). After purification, the DNA was cleaved with restriction enzymes, and specific fragments were purified on 1.4% alkaline agarose gels. Bands were visualized by autoradiography or ethidium bromide staining, cut out of the gel, and electroeluted. The eluted DNA was phenol extracted once and stored as an ethanol precipitate at −20°C.

Preparation of RNA. Cells were infected for 48 h with 20 PFU of virus per cell. In mixed infections the inocula consisted of 20 PFU of each type of virus per cell. The cytoplasmic and nuclear RNAs were prepared as previously described (14). Nuclear fractions were digested with RNase-free DNase prepared by previously described procedures (13). The DNase treatment of cytoplasmic fractions was found not to be necessary for the nuclease S1 hybridization experiments.

Nuclease S1 analysis. Nuclease S1 hybridization analysis was performed using DNA probe excess with 80% formamide hybridization conditions as described previously (3). Samples were electrophoresed on 30-cm 5 or 8% acrylamide-bisacrylamide (40:1) gels containing 7 M urea. Gels were autoradiographed at −70°C on Kodak XAR-5 X-ray film with DuPont Cronex Lightning-Plus intensifying screens. In these experiments it was found that a hybridization temperature of 53.3°C gave optimal results.

Analysis of viral proteins. Proteins were labeled with [^{35}S]methionine, immunoprecipitated, and analyzed on sodium dodecyl sulfate-polyacrylamide gels as previously described (1).

RESULTS AND DISCUSSION

Description of the viruses. The wild-type strain used in these experiments was 776. In addition, three viable deletion mutants were used. Mutant *dl*861 (4, 6) is missing 16 to 25 nucleotides at the *Hpa*II site (see Fig. 1) within the late mRNA leader region, resulting in removal of the translational start codon for the agnoprotein (10). The position of the deletion shown in Fig. 1 is based on nuclease S1 results from this laboratory. The precision to within two bases on the downstream side of the *Hpa*II site is quite certain; the dotted area on the upstream side shows some uncertainty in the location of this end of the deletion.

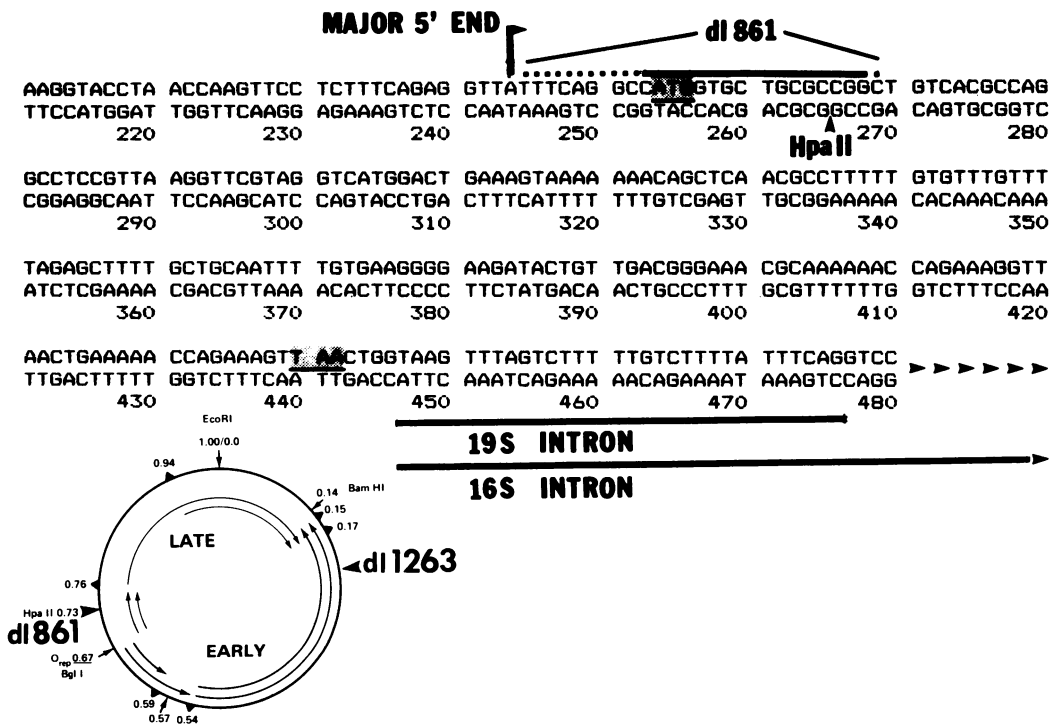

FIG. 1. Sequence of the major late leader region of SV40 DNA and genomic map. The major 5' end of the SV40 late mRNAs is shown; this is also the beginning of the major 202-nucleotide leader which ends at the introns for the 19S and 16S mRNAs. The *Hpa*II site is noted; this is the site used for the construction of *dl*861. The tentative map location of the *dl*861 deletion is shown as determined by nuclease S1 analysis; the solid line indicates bases which are certainly deleted, and dots indicate bases which may be deleted as well. The shaded bases indicate the translation short and stop codons for the agnoprotein. Nucleotide numbering is the modified Reddy numbering (12a). The genomic map shows all landmarks important for this paper.

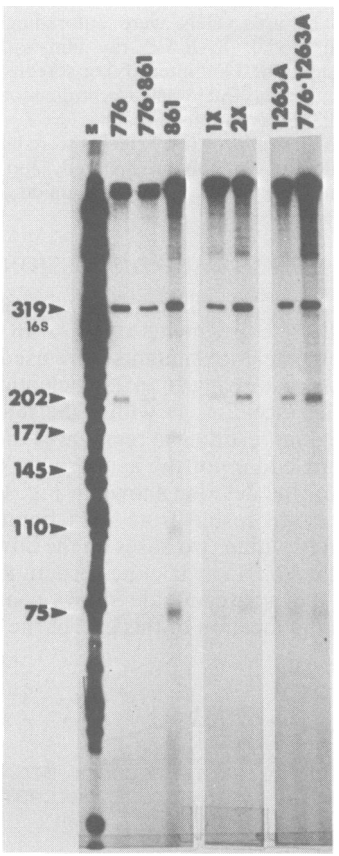

FIG. 2. Nuclease S1 analysis of cytoplasmic SV40 late mRNAs. Total cytoplasmic RNA was extracted from cells lytically infected with SV40 for 48 h. RNA (2 μg) was analyzed with a ^{32}P-labeled SV40 DNA probe extending from the *Bgl*I site to the *Eco*RI site (see Fig. 1 and the text). Lanes: M, DNA size markers; 776, RNA from cells infected with wild-type 776; 776-861, RNA from cells infected with both wild-type 776 and *dl*861; 861, RNA from cells infected with *dl*861; 1X, RNA from cells infected with 20 PFU of 776 per cell; 2X, RNA from cells infected with 40 PFU of 776 per cell; 1263A, RNA from cells infected with *dl*1263A; 776-1263A, RNA from cells infected with wild-type 776 and *dl*1263A.

*dl*1263 contains the same deletion as *dl*861; thus, all of the effects described below when *dl*861 was used also occurred when *dl*1263 was used. In addition, *dl*1263 contains a 33-base pair deletion at 0.21 map units (m.u.) in the early coding region (see Fig. 1). This deletion results in the production of a large T-antigen which is smaller than wild type (5). This difference can be used to differentiate between mutant and wild-type early expression. Mutant *dl*1263A was constructed in this laboratory by removing the *dl*861 deletion from *dl*1263 and replacing it with wild-type sequences (the fragment between the *Bgl*I site, 0.67 m.u., and the *Hae*II site, 0.83 m.u., was replaced). *dl*1263 was used in this reconstruction to ensure that the isolate was not made up of ligated pieces of wild-type DNA.

Results of mixed infection. Figure 2 shows the nuclease S1 analysis of late viral RNAs in the total cytoplasmic fraction from CV-1P cells lytically infected with either 20 PFU of 776 per cell, 20 PFU of *dl*861 per cell, or 20 PFU each of 776 and *dl*861 per cell. The DNA probe used in these experiments was the fragment from the *Bgl*I site, 0.67 m.u., to the *Eco*RI site, 1.00 m.u. (see Fig. 1). This probe and an 8% polyacrylamide gel were used for analysis. The viral RNAs most readily identified in the wild-type infection (lane 776) are represented by a 319-nucleotide band, resulting from the presence of the 16S late viral mRNA body, and a 202-nucleotide band, resulting from the major late leader (see also Fig. 1 and 4). The band corresponding to the 19S late mRNA body migrated at the top of the gel and is not well resolved from the reannealed probe. Examination of the nuclease S1 results involving deletion mutant RNA (lane 861) shows that the 202-nucleotide leader band is reduced in size to 177 nucleotides due to the deletion. In addition, several smaller leaders are used more prominently by the deletion mutant than by the wild type; this has been observed by others (12a). The most interesting result in this experiment was that obtained with the mixed infection (lane 776-861). It was observed that, compared with infection by either virus alone, there is not only a decrease in the 16S (319-nucleotide) band and in the wild-type leader, but there is also no evidence at all of RNAs from the mutant virus, as indicated by the absence of the mutant-derived leaders. Thus, a *trans* effect, resulting in a decrease or disappearance of late transcripts, is indicated in the mixed infection.

To insure that these decreases were not due to the doubling of multiplicity which occurs in the mixed infection, cultures were infected with 20 or 40 PFU of wild-type or *dl*861 per cell, and the total cytoplasmic RNA was analyzed as above. In both cases, the doubling of the multiplicity resulted in an equivalence of or an increase in late RNAs rather than the decrease observed in the mixed infection. This is shown for the wild-type infection in lanes 1X and 2X of Fig. 2. The results suggest that the *trans* effect occurring in the mixed infection is not due to multiplicity, but is due to the mixture of a wild-type strain with a strain containing the *dl*861 deletion. To demonstrate that this effect was due to the presence of the *dl*861 deletion, the deletion was removed in the construction of *dl*1263A described above. This mutant contains the early region deletion of *dl*1263 but has had the *dl*861 deletion replaced

with the wild-type sequence. A mixed infection between wild-type 776 and *dl*1263A no longer resulted in decreased levels of cytoplasmic late mRNA (compare lanes 1263A and 776-1263A in Fig. 2). Thus the *trans* effect appears to be due to the presence of the *dl*861 deletion in the mixed infection.

Effect of the *dl*861 deletion on early gene expression and replication in mixed infections. Figure 3 shows the results of experiments to examine the effect of the *dl*861 deletion on early gene expression and viral DNA synthesis in mixed infections with wild-type virus. Figure 3 (lanes headed "protein") shows a sodium dodecyl sulfate-polyacrylamide gel of [^{35}S]methionine-labeled viral proteins immunoprecipitated with antisera which recognized large and small T-antigens, as well as the major coat protein VP-1. *dl*1263 was used in this mixed infection because it contains both the *dl*861 deletion and a deletion in the early region which causes a smaller large T-antigen to be produced. This difference allows early gene expression from each genome to be detected in the mixed infection. Clearly, both forms of large T-antigen are being synthesized in the mixed infection. In addition, the amount of VP-1 precipitated from the mixed infection shows that despite the reduction in 16S RNA (Fig. 1), relatively large amounts of VP-1 can still be synthesized.

Viral DNA synthesis was examined by restriction analysis of ^{32}P-labeled DNA. Since the deletion in *dl*861 was introduced at the *Hpa*II site, this DNA can be distinguished from wild type by the lack of *Hpa*II cleavage. The labeled DNA from infected cultures was cleaved with *Hpa*II, *Bam*HI, and *Eco*RI (see Fig. 1). These digestions cleave wild-type DNA into three fragments of 3,056, 1,436, and 751 nucleotides (Fig. 3), whereas the deletion in *dl*861 results in fusion of the 3,056- and 1,436-nucleotide fragments, forming a 4,492-nucleotide fragment. Clearly, both DNAs are being produced in the mixed infection.

The above results indicate that the presence of the *dl*861 deletion in a mixed infection has no substantial effect on major events in the lytic cycle other than late RNA synthesis. This is, however, not an exhaustive examination of viral functions. Later viral functions, such as packaging, may be affected by the deletion; however, this seems unlikely since progeny titers of single and mixed infections are relatively equal, and the progeny of an equally mixed infection show equal levels of synthesis of each genome in subsequent infections (data not shown). This suggests relatively equal production of each virus during the original mixed infection and indicates that packaging was equivalent for each virus.

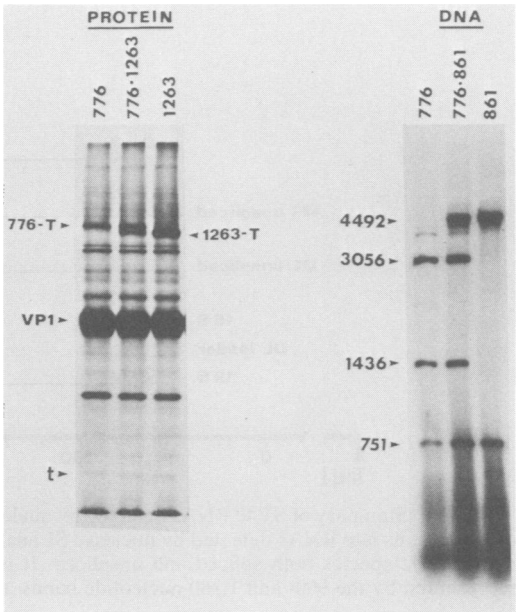

FIG. 3. Effect of mixed infection on early gene expression and replication. [^{35}S]methionine-labeled proteins or ^{32}P-labeled DNA was isolated from cells infected with: wild-type 776 (lanes marked 776); a mixture of wild-type 776 and either *dl*1263 or *dl*861 (lanes marked 776-1263 or 776-861); *dl*861 (lanes marked 861). Labeled proteins were immunoprecipitated with antisera against large and small T-antigens as well as VP-1. Gel analysis was on a 10% sodium dodecyl sulfate-polyacrylamide gel (see the text). The [^{32}P]DNA was isolated by the Hirt procedure (9) without banding in cesium chloride-ethidium bromide gradients. The DNA was cleaved with the restriction endonucleases *Hpa*II, *Bam*HI, and *Eco*RI. Gel analysis was on a 1.4% agarose gel.

Examination of nuclear transcripts. The mixed-infection data in Fig. 2 suggest that in the cytoplasm the amount of wild-type late transcripts is reduced, and mutant transcripts are undetectable. To determine what may be happening in the mixed infections, the nuclear RNAs were examined by nuclease S1 analysis. Due to the deletion in *dl*861, the major unspliced precursor transcripts from mutant and wild-type virus are distinguishable by nuclease S1 analysis (see Fig. 4). Figure 5 shows the results of analysis of cytoplasmic and nuclear RNAs, using a hybridization probe which extends from the *Taq*I site (0.57 m.u.) to the *Eco*RI site (1.00 m.u.). This probe was chosen because it is larger than any expected band; thus, a reannealed probe will not interfere with interpretation. Comparing the cytoplasmic and nuclear samples, the large nuclear precursors can clearly be

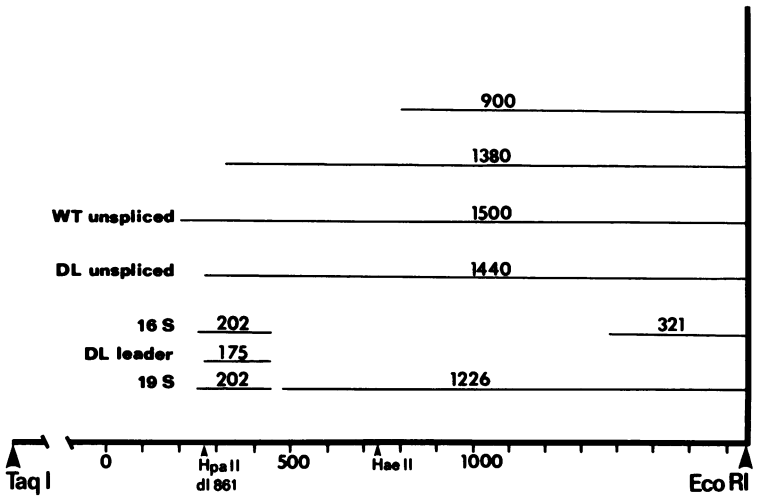

FIG. 4. Summary of SV40 RNAs detected by nuclease S1 analysis. Shown are the approximate map positions of the various late RNAs detected by nuclease S1 analysis in these experiments. The RNAs include nuclear and cytoplasmic species both spliced and unspliced. It is not known from these experiments whether the RNAs represented by the 900- and 1,380-nucleotide bands are spliced.

seen. The mapped locations of the 5′ ends of all of the late transcripts, determined by nuclease S1 analysis (data not shown), are diagrammed in Fig. 4. Among the late RNAs detected, the 1,500- and 1,440-nucleotide bands represent the major unspliced late transcripts of the wild type and *dl*861, respectively (8, 11). Other late RNAs detected in the nucleus are indicated by the 1,380- and 900-nucleotide bands. The 900-nucleotide species has been recently reported by Villarreal (14) as being a minor species tightly associated with the perinuclear fraction; in these experiments, this RNA was found abundantly in the cytoplasm. There is presently no explanation for these differences between the two experiments regarding the 900-nucleotide species. Examination of the nuclear RNAs from the mixed infections (Fig. 5A and B) revealed that the wild-type 1,500-nucleotide band was present but re-

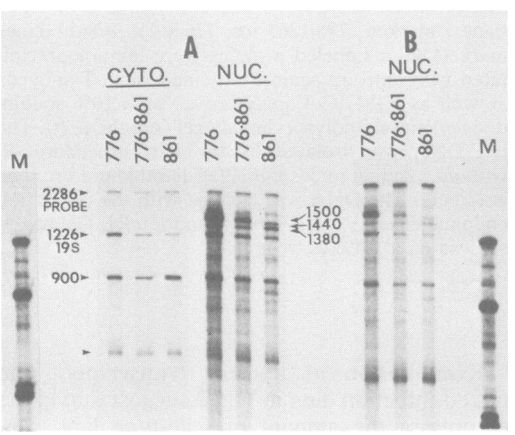

FIG. 5. Nuclease S1 analysis of unspliced nuclear precursors of SV40 late mRNA. Nuclease S1 analysis was performed as described in the legend to Fig. 2; however, a [^{32}P]DNA probe was used which extended from the *Taq*I site to the *Eco*RI site. (A) Analysis of cytoplasmic and nuclear RNAs from cells infected with wild-type 776, *dl*861, or a mixture of 776 and *dl*861. (B) Second analysis of nuclear RNA with a lighter exposure of the gel. The major band migrating below the 900-nucleotide species is an S1 antifact found in the analysis of uninfected-cell RNA. Note that the amount of 19S RNA and precursor is less in the mutant than in the wild type. This phenomenon, in relation to the relatively equal amounts of 16S RNA shown in Fig. 2, may indicate differences in the ratios of late RNAs as well as transcriptional deficiencies caused by the *dl*861 deletion.

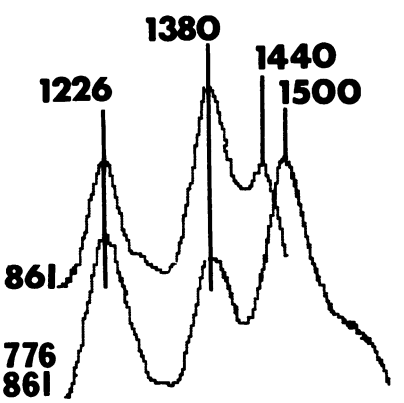

FIG. 6. Microdensitometer scan of the unspliced precursor bands shown in Fig. 5. Lanes 776-861 and 861 in Fig. 5A were scanned. The positions of the peaks in lane 776 (Fig. 5) superimpose on the scan of lane 776-861. Similar scans were generated from the data in Fig. 5B.

duced (compared with 776 alone), and the 1,440-nucleotide band, indicative of mutant late transcript, was undetectable. To clarify this observation, a microdensitometer scan is shown in Fig. 6; no evidence of the 1,440 band is seen in the mixed infection. Clearly, the major late nuclear precursor from the mutant genome is greatly reduced or nonexistent in the mixed infection. These nuclear results suggest that the mixed infection effect occurs at the transcriptional level.

Obviously, the mixed-infection effect (the reduction of wild-type late transcripts with the apparent loss of detectable mutant transcripts) is occurring due to an aberrant *trans*-acting interaction between the wild type and *dl*861. The *trans* action suggests that this is mediated by a diffusible factor. The data support a variety of models for the mechanism of the effect; the model presented below is by no means conclusive, but at this point it offers an explanation which accounts for the entire phenomenon. No matter what the exact mechanism, this aberrant interaction has revealed a heretofore uncharacterized level of SV40 late gene expression control. The nuclear data in Fig. 5 suggest that this may be occurring at the transcriptional level since the major nuclear precursors of late RNA are affected. Within the context of the data, a model can be proposed involving positive control. Assume that the diffusible factor exerts its effect by interaction with sequences deleted in *dl*861 and is encoded within these sequences. The *dl*861 deletion would then eliminate the factor, as well as the need for it; thus, the virus is viable and appears, by most criteria, to be very similar to wild type. However, if the diffusible control factor is present, as in the mixed infection, it may now interact with the mutant in an aberrant fashion. Instead of performing its normal function, the factor may interact irreversibly with the mutant, resulting in disruption of late transcription. Since mutant genomes continue to be made (Fig. 3), the factor would continue to be bound up. If the factor normally exerts positive control on wild type, then the aberrant interaction of factor with mutant would lead to the prediction that wild-type late transcripts should be reduced, since there is a reduced, active concentration of factor. This is what has been noted in all of the experiments. Another prediction of this model is that changing the ratio of viruses in the mixed infection should alter the amounts of reduction of either type of transcript; this has been noted in several experiments (data not shown).

Overall, the data indicate a heretofore uncharacterized transcriptional control for at least the major late primary transcripts. Predictions from a model proposing a diffusible positive control factor are supported. The deletion in *dl*861 removes the translational start codon for the agnoprotein (10; Fig. 1). Thus, the putative factor may be the agnoprotein, a particularly interesting possibility since the agnoprotein demonstrates DNA-binding properties (10) which would fit the model.

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