

## Role of the Agnoprotein in Regulation of Simian Virus 40 Replication and Maturation Pathways

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**Analysis of two agnogene mutants, *dl2304* deleted over the entire agnogene and *in2379* carrying a 2-base insert, indicated that the mutant phenotype of small plaque formation must be the result of a defect late in the maturation pathway. Both mutants were removed from the pool of molecules available for replication with wild-type kinetics. Whereas *dl2304* was somewhat reduced in its rate of progression from chromatin to previrions-virions, *in2379*, which produced even smaller plaques than *dl2304* did, progressed with wild-type kinetics. Therefore, the agnoprotein was not required for progression from chromatin to previrions.**

The late leader region of simian virus 40 (SV40) contains an open reading frame (agnogene) which encodes a small polypeptide (4). This polypeptide, called the agnoprotein or leader protein 1, is a 7.9-kilodalton basic protein with affinity for single- and double-stranded DNA (11, 13). Using immunofluorescence, Nomura et al. (20) showed that the protein is predominantly located in the cytoplasm and perinuclear region. Mutations in the leader region result in production of smaller plaques, indicating that something has slowed in the normal replication cycle but that this function is not absolutely required for growth in tissue culture cells (9, 15, 16, 23, 24). Evidence has been presented suggesting that the agnogene and the agnoprotein act as regulators of structural protein synthesis (1, 10). In addition, pseudorevertants of VP1, the major structural protein, map in the agnogene, suggesting an interaction between these two proteins (14). Whereas this observation is consistent with a role for the agnoprotein in the regulation of gene expression, it could indicate that the agnoprotein plays a role in the maturation pathway (13, 14, 16). Models which involve the agnoprotein in the latter process must take into account that the agnoprotein is not detected in virions (11, 13).

We recently reported that a *cis*-acting sequence which is located in the *Hind*III E fragment of SV40 (the overlap between VP2 and VP3) is necessary for efficient removal of DNA from the replication pathway (26). In the absence of this sequence, newly synthesized DNA is used as a template for further DNA synthesis to a greater extent than is wild type DNA. The proteins which interact with this sequence are not known. We have developed a protocol to analyze the involvement of SV40 DNA in the replication and maturation pathways (27). Removal of DNA coincides temporally with the progression of DNA from the chromatin pool to the previrion-virion pool. To gain insight into the function of the agnoprotein, we analyzed the effect of two independent mutations in the agnogene on these two pathways. *dl2304* is deleted over the entire agnogene (13); *in2379* contains a 2-base-pair insert within the *Hpa*II site in SV40 (19) (Fig. 1). This latter insertion results in a frameshift, giving rise to a truncated agnoprotein of 33 instead of 61 amino acids; only the first 5 are identical to those of the wild-type protein. However, the *in2379* agnoprotein is not detectable and therefore, if produced, must be much less stable than the

wild-type agnoprotein (19). These two mutants were chosen for study because *dl2304* also has an altered ratio of 16 to 19S mRNAs, whereas the ratio in *in2379* is similar to that of the wild-type (G. Khoury, personal communication). Both of these mutants produce small plaques on indicator monkey kidney cells (19; data not shown), as is true for other agnogene mutants (9, 15, 16, 24). The *in2379* plaques are smaller than the *dl2304* plaques (19; data not shown).

To determine whether the agnoprotein plays a role in removing DNA from the replication pathway, SV40-infected CV-1 cells were pulse-labeled for 20 min with [<sup>3</sup>H]thymidine, chased for 30 min with cold thymidine, and then chased in medium containing bromodeoxyuridine (BUdR) (27). If molecules replicated during the pulse (LL-labeled DNA) are used as templates for further rounds of replication, then BUdR will be introduced into the other strand and the DNA duplex will have [<sup>3</sup>H]thymidine in one strand and BUdR in the other (HL-labeled DNA). Thus, to monitor the involvement of the pulse-labeled DNA in the replication pathway, the percentage of HL-labeled DNA was determined based on counts per minute (cpm) as follows: percent HL DNA =  $\text{cpm HL} / (\text{cpm HL} + \text{cpm LL}) \times 100$ . Molecules labeled during the pulse are said to reenter replication if they are used as templates for further DNA synthesis during the chase. Therefore, the kinetics of reentry of pulse-labeled DNA into replication was determined with increasing time in the BUdR chase mixture as a function of the status of the agnoprotein. The results of a representative experiment are shown in Fig. 2. As shown previously (27), wild-type 776 (wt776) DNA was used as a template for further DNA synthesis for a limited period of time and then was removed from the pool of molecules available for replication (as illustrated by the leveling off of the curve). If the agnoprotein were responsible for removing DNA from the replication pathway, in the absence of the agnoprotein the percentage of HL DNA should continue to increase. However, the same kinetics of reentry were seen with the two agnogene mutants as with wild-type. As stated above, removal of DNA from the replication pathway appears to involve interaction of a *cis*-acting removal sequence (located at least 500 base pairs away from the agnogene in the late side of SV40) with a putative protein. The agnoprotein is not involved in this step, because the removal seen with both mutants is the same as with wild-type.

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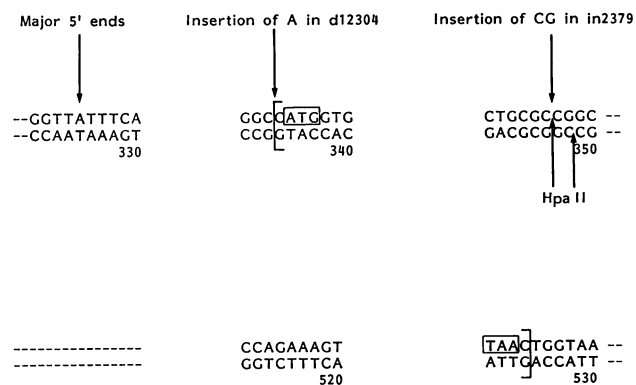


FIG. 1. Location of the *dl2304* and *in2379* mutations. The major 5' ends of 16 and 19S mRNA are shown. The ATG and TAA (boxed) are the start and stop codons for the agnoprotein. The large brackets show the beginning and end of the *dl2304* deletion; an A is inserted as shown (G. Khoury, personal communication). The 2-base insertion in *in2379*, indicated by the upper arrow, converts the *Hpa*II site to an *Sst*II site. The *cis*-acting removal sequence is located between nucleotides 1046 and 1494 (26). The numbering system is that of Tooze (25).

To determine whether agnoprotein plays a role in the conversion of chromatin to previrions, nucleoprotein complexes were isolated from cultures labeled and chased as described above. The complexes were fractionated on sucrose gradients which allowed the separation of chromatin from previrions-virions but not 220S previrions from salt-stable virions (2, 3, 5–7, 27). The involvement of pulse-labeled DNA in the maturation pathway was determined by plotting the percentage of <sup>3</sup>H-labeled previrions-virions with the time of the chase [percentage of <sup>3</sup>H-labeled previrions-virions = (previrion-virion cpm)/(total nucleoprotein complex cpm) × 100]. (That BUdR was present in the chase is irrelevant to this part of the experiment). The results of a representative experiment are shown in Fig. 3. Approximately 60% of wt776 DNA progressed from chromatin to previrions-virions. If the agnoprotein were responsible for the progression from chromatin to previrions, in the absence of the agnoprotein the percentage of previrions-virions should be less than that seen with wt776. *dl2304* showed a slowed progression to previrions-virions when compared to wt776. In contrast, progression of *in2379* chromatin to previrions-virions occurred with wild-type kinetics. These data indicate that the agnoprotein is not required for transition from chromatin to previrions. Taken together, the data on the two mutants indicate that the slow progression seen with *dl2304* is independent of the agnogene product. In fact, the magnitude of the difference in extents of maturation between wt776 and *dl2304* seems to be insufficient to completely account for the defect in *dl2304*. In a one-step growth curve, *dl2304* yielded 1/10 the titer of wt776 (data not shown). The maturation data further suggest that the absence of the agnoprotein has a major impact late in the maturation pathway (noted by plaque size). *dl2304* has an additional defect, detected by the kinetic analysis in Fig. 2, which is discussed below.

Although (in contrast to *dl2304*) *in2379* progressed into the maturation pathway with wild-type kinetics, the plaques formed by *in2379* were smaller than those produced by *dl2304* (data not shown). The critical nature of the *in2379* mutation was further supported by its instability. During the course of the experiments described here, it became clear

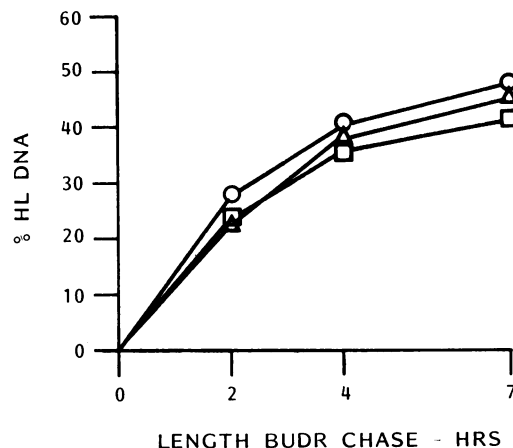


FIG. 2. Reentry kinetics of *dl2304*, *in2379*, and wt776. Infected cells were labeled as described in the text, and the percentage of [<sup>3</sup>H]DNA present in HL DNA was determined. Symbols:  $\Delta$ , wt776;  $\circ$ , *dl2304*;  $\square$ , *in2379*.

that the *Sst*II site generated in *in2379* by the insertion was lost at a high frequency (data not shown). Therefore, the mutant genome was propagated in bacteria after being cloned into pBR322. The *in2379* DNA was then recovered and used to produce a virus stock in CV-1 cells. All of the yield could be cut with *Sst*II. This stock was used in the experiments shown in Fig. 2 and 3, and the recovered DNA was also susceptible to *Sst*II. The experiments were, in fact, conducted with CV-1 cells because *Sst*II-resistant DNA was detected after a single passage in BSC-1 cells. A difference in the behavior of SV40 mutants in these two cell lines has also been reported for two mutants in T antigen (21). Nomura et al. (19) recently reported that *in2379* is unstable and grows less well than mutants carrying small deletions in this region.

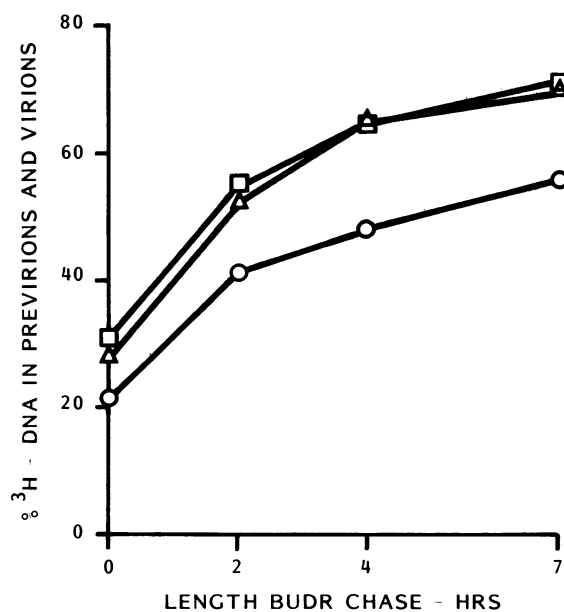


FIG. 3. Kinetics of maturation of *dl2304*, *in2379*, and wt776. Infected cells were labeled as described in the text, and the [<sup>3</sup>H]DNA in chromatin and in previrions-virions was determined. Symbols:  $\Delta$ , wt776;  $\circ$ , *dl2304*;  $\square$ , *in2379*.

Resnick and Shenk (22) attributed the tiny plaques seen in the mutants carrying the 2-base insertion to the fact that the insertion not only inactivates the agnogene but also shifts the agnogene reading frame into frame with VP2, resulting in a VP2 gene with additional codons derived from the agnogene region (but out of frame with the agnogene).

Ng et al. (18) determined the maturation kinetics of two other agnogene mutants. One of the mutants examined, *dl805*, is deleted from nucleotide 335 through 517 (16) and is therefore similar to *dl2304*. The progression of *dl805* chromatin to previrions-virions was slow compared to that of wild-type. *dl810* that had been deleted from nucleotides 297 to 471 (16) also progressed more slowly to previrions-virions (18). Because the levels of VP1, VP2, and VP3 for both mutants were near normal (18), these data were interpreted to suggest that the agnoprotein plays a role in maturation from chromatin to virions. This transition involves the buildup of protein around the chromatin core (3, 5, 8, 12, 17). The data presented here indicate that the agnoprotein is not involved in the transition from chromatin to previrions. Consistent with this latter interpretation, Resnick and Shenk (22) showed that conversion of the agnogene initiation codon from ATG to TTG prevents synthesis of the agnoprotein but does not alter maturation kinetics. The loss of a *cis*-acting element in *dl2304*, *dl805*, and *dl810* could explain the slow maturation of these mutants. However, Mertz et al. (16) showed that *dl810* can be complemented by the other SV40 complementation groups. Perhaps the complementation assay measures the major role for the agnoprotein late in maturation but does not detect the additional defect revealed by kinetic analysis of the progression of chromatin to previrions.

In summary, the defect in the agnogene mutants appears to be late in the maturation pathway after removal of DNA from the replication pathway and its progression to previrions. The mutant phenotype (smaller plaques in tissue culture) may be due to some extent to the absence of the agnogene product (e.g., *dl2304*). The still smaller plaques seen in *in2379* and the propensity of *in2379* to mutate suggest that the 2-base insertion has further negative ramifications.

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