Activity of Simian Virus 40 Late Promoter Elements in the Absence of Large T Antigen: Evidence for Repression of Late Gene Expression

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We used chloramphenicol acetyltransferase transient expression to examine the activity of the promoter elements of the simian virus 40 late promoter in the absence of large T antigen. Since the experiments were done in permissive CV-1 cells, these conditions mimic the state which exists early in the viral lytic cycle before the onset of replication and T-antigen-mediated trans activation. Our data, using deletion analysis, indicate that removal of the 21-base-pair (bp) repeat region causes as much as a 10-fold increase in activity of the late promoter elements. This result suggests that the 21-bp repeat sequences may be involved in repression of the late promoter elements during the early phase of the lytic infection. This is supported by competition analysis which indicates that increasing amounts of competitor containing only the 21-bp repeat region results in increased activity of the intact promoter. A model for the activity of the late promoter through the course of lytic infection is presented.

The activation of the simian virus 40 (SV40) late promoter has been shown to occur by a trans activation mechanism mediated by the viral early protein, large T antigen (1, 3, 4, 17, 18). Part of this activation can occur in the absence of both viral DNA replication (3, 7, 17, 18) and the T-antigen binding sites within the viral origin of replication (7, 18). Several laboratories, including ours, have undertaken studies to determine the elements of the late promoter which are necessary for trans activation to occur (4, 7, 15, 18). Figure 1 shows elements of the late promoter previously defined by mutational analyses done in this laboratory (18); they are designated omega (ω), delta (δ), and tau (τ). It should be noted that these elements overlap the defined elements of the early viral promoter (the guanine-plus-cytosine-rich 21-base-pair [bp] repeats and the 72-bp repeat enhancer region) and the T-antigen binding sites (I, II, and III) within the region of the origin of replication. The delta element, SV40 nucleotides 200 to 270 (nucleotide numbering is described in reference 25), is active in the presence of T antigen only when the origin region, the omega element, is intact. The delta-omega combination accounts for approximately 35% of the late promoter activity when the wild-type promoter is in the presence of T antigen (18). The tau element (SV40 nucleotides 150 to 200) requires neither an origin region nor the T-antigen binding sites to cause late promoter activation in the presence of T antigen. Under wild-type conditions the tau element contributes approximately 65% of the late promoter activity (18). The tau element also has been shown to demonstrate an orientation dependence (18). Overall, these data indicate two means of activating the late promoter.

Because the delta element appears to require the specific T-antigen binding sites at the origin (and possibly the act of replication), we feel that the activation mechanism mediated by the delta element may be specific to the SV40 system. Conversely, the independence of the tau element of the T-antigen binding sites suggests that it may be representative of more general trans-activatable elements which do not require direct T-antigen binding for their activation. Indeed, a variety of evidence (1, 2, 20, 23), has led to the suggestion (18) that promoter activation mediated through the tau element is not a function of the direct interaction with T antigen. Instead, activation of the tau element may be mediated by a cellular trans-acting factor(s) which is induced, activated, or modified due to the presence of T antigen.

In the present study we analyzed the activity of these promoter elements, and surrounding regions, under nonactivated conditions, i.e., in the absence of T antigen, to determine which elements direct the low levels of late promoter activity detected early in the lytic infection and in nonpermissive cells (6, 8, 19). These studies yielded the unexpected result that the guanine-plus-cytosine-rich regions of the 21-bp repeats may be a site involved in the repression of late transcription during the early phases of infection. In an earlier publication (18) we noted that this region affects late promoter activity; however, the effect was not fully characterized. At that time we called the region element II of the late promoter. In the present paper we call it the rho (ρ) element to avoid confusion with T-antigen binding site II.

MATERIALS AND METHODS

Transfection and CAT analysis. The studies of the promoter activity were performed in chloramphenicol acetyltransferase (CAT) transient-expression vectors (10). The specific plasmid constructs with the SV40 late promoter, and deletions within it, have been previously described (18) and are shown in Fig. 2. CV-1P cells, an established line of African green monkey kidney cells, were transfected with the plasmids (5 μg/5 × 10⁶ cells on a 60-mm tissue culture dish) by using the calcium phosphate precipitation procedure as previously described (10, 11, 18). The cells were harvested 40 h posttransfection. One-half of the cells was extracted for CAT assay (10, 18); the other half was extracted by the method of Hirt (16) and analyzed by quantitative Southern blotting (22) to determine the amount of plasmid DNA in the cells at the time of harvest. This

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quantitation was used to standardize the CAT activities to DNA copy number. This standardization procedure has been shown to be an effective means to correct for variations in transfection efficiencies (1). Standardized CAT activities were converted to units of CAT activity by comparison to a standard curve of CAT activity generated by using known amounts of purified CAT (P-L Biochemicals, Inc.). As in our previous studies (1, 18) each activity was also standardized to reflect the activity generated by 10⁶ transfected cells; cell counts were determined from a mock-transfected plate. Thus, the data in Fig. 2 are expressed in standardized microunits of CAT activity per 10⁶ transfected cells.

**Competition analysis.** Competition analysis was performed by cotransfecting cells with the test plasmid, pL16 (5 μg per plate), and increasing amounts (5, 10, and 15 μg) of competitor plasmid. The competitor used was p21, a plasmid derived from pL9-cat, a late promoter CAT plasmid which has the CAT gene located at nucleotide 108, just to the late side of the 21-bp repeats; the junction between the promoter and the CAT gene was constructed with a SalI linker. By cleaving with SalI and EcoRI (the EcoRI site is within the CAT gene) most of the CAT gene was deleted. After ligation the resulting plasmid was cleaved with HindIII (at the junction of the plasmid sequences and the late promoter insert, SV40 nucleotide 5171; Fig. 1) and NcoI (cleaving at SV40 nucleotide 37, within the late promoter insert) removing all SV40 promoter sequences except the region of the 21-bp repeats (specifically, nucleotides 37 to 108). After ligation the resulting plasmid was an appropriate competitor containing only the 21-bp repeat region and a deleted, nonfunctional CAT gene. A total promoter competitor was prepared by cleaving pL16 with SalI and EcoRI. After ligation the resulting plasmid retains the intact late promoter but a deleted, nonfunctional CAT gene. Additional competition experiments were performed with the Rous sarcoma virus long terminal repeat; here the competitor plasmid was pRSV-neo (11).

**Northern blot analysis.** The levels of CAT-specific RNA generated from the various late promoter-CAT plasmids, in the absence of T-antigen-mediated trans activation was determined by Northern blot analysis with [α³²P]RNA probe specific for CAT sequences. Cells were transfected as described above, but a larger number of cells was used (one T150 tissue culture bottle per transfection) to prepare enough RNA to easily detect. The Northern blot analysis utilized one-fifth of the polyadenylated RNA fraction isolated from each T150 flask. A small amount of the transfected cells from each transfection was used for quantitation of plasmid DNA (see description above) to determine any variations in transfection efficiencies among the various samples.

**RESULTS**

**Activity of the late promoter elements in the absence of T antigen.** The fragment of SV40 DNA (SV40 nucleotides 5171 to 333) which forms the promoter region of the CAT vector pL16 (17, 18) is shown in Fig. 2. For the studies presented here this region represents the wild-type promoter. This fragment contains an intact origin of replication; thus, pL16 and all other plasmids containing the origin of replication region are replicative and will replicate if T antigen is present (17, 18). Nonreplicative counterparts of each plasmid were generated by introducing a 6-bp deletion at the BglII site within the origin of replication. This deletion has been previously shown to abolish the replicative capacity of the origin (9, 17, 18). Both replicative and nonreplicative (Fig. 2, Rep. and Nonrep.) plasmids were evaluated for promoter activity in the absence of trans activation by T antigen.

The wild-type plasmid pL16 generated the expected low level of promoter activity (5 to 10 μU/10⁵ cells; Fig. 2). For
comparative purposes, under conditions of T-antigen-mediated trans activation the activities of the plasmids listed in Fig. 2 are 10 to 1,000 times greater than their respective activities shown in Fig. 2, depending on the specific deletion and whether the plasmid is replicative or nonreplicative (18). The activities of the various plasmids in Fig. 2 appear to be small; this is a consequence of the standardization to reflect the activity generated by 10⁵ cells. This standardization makes the data comparable to previous results (18). The actual assays of CAT were performed with sufficient amounts of transfected cell extract to produce results within the linear range of the CAT assay. Thus, the activities shown are accurate, and the differences in activities are significant.

Deletion of the tau and delta elements in pL163 caused little change in the promoter activity, suggesting that these promoter elements are relatively inactive in the absence of trans activation and that the promoter activity detected is generated by elements located between the origin of replication and tau. In this regard, we have previously noted that under trans-activated conditions the 21-bp repeat region can generate a low level of promoter activity, but this activity is detected only when the 72-bp repeat region is deleted (18).

The promoter activities generated by plasmids pL533, pL540, pL561, and pL563 suggest that the inactivity of the tau and delta elements may be due to repression. In pL533 and pL540 the 21-bp repeat region was deleted resulting in as much as a 10-fold increase in promoter activity in both replicative and nonreplicative forms. That the increased activity was generated by the tau and delta elements is indicated by comparing the activities of pL540, pL561, and pL563. The deletion of the tau element (compare pL540 with pL561) caused the loss of most of the increased activity when the plasmids were in the nonreplicative form. However, this deletion did not affect CAT activity in the replicative form of pL561. Instead, the increased activity was lost in the replicative plasmids only when both the tau and delta element were deleted (compare replicative pL561 with pL563). This difference indicates that the integrity of the origin has an effect on the basal (non-T-antigen activated) function of these elements. This is supported by the assay of pLT1h and pLT1l; in pLT1l the entire origin region and the 21-bp repeats are deleted, but the tau and delta elements remain intact. Under these conditions the tau and delta elements do not generate activities comparable to the nonreplicative pL533, again suggesting that features of the origin region must be preserved for the basal activity of both tau and delta elements in the absence of T antigen. However, the nature of these origin of replication region features are unclear at this point since a deletion of only 6 bp at the BglII site (as in the nonreplicative plasmids) and the deletion of the entire origin of replication region (pT1l) have very different effects (compare nonreplicative pL533 with pT1l).

Northern blot analysis of CAT-specific RNA. The most significant finding in the above data is that in the absence of T-antigen-mediated trans activation, the deletion of the 21-bp repeat region causes a significant increase in the basal activity of the late promoter elements. To verify that the CAT data truly reflect differences in the levels of RNA, Northern blot analysis was performed. Identical transfections were performed but with a larger cell number to produce a greater quantity of RNA for detection (see above). Figure 3 shows the results of this analysis with a [³²P]RNA probe specific for CAT gene sequences. In agreement with the CAT enzyme results, the plasmids with the 21-bp repeat region (rho element) deleted make substantially more RNA than does pL16. Densitometric quantitation suggests approximately a 10-fold difference when standardized for differences in transfection efficiencies. RNA analyses of the transcripts generated from these late promoter-CAT vectors have also been presented previously (17, 18). In agreement, these previous analyses show that CAT mRNA

FIG. 3. Northern blot transfer analysis of CAT-specific RNA from CV-1P cells transfected with late promoter CAT plasmids. Equivalent amounts of polyadenylated RNA isolated from transfected cells were utilized (see the text). Mock-transfected cell RNA was derived from CV-1P cells transfected with pSV0-cat (10). The probe was a CAT-specific [³²P]RNA (see the text).

FIG. 4. Late promoter activity in competition transfection experiments. CV-1P cells were transfected with equal amounts of pL16-cat and various amounts of plasmids containing competitor promoters or promoter elements. The p element, late promoter, and Rous sarcoma virus long terminal repeat (RSV-LTR) competitor plasmids are described in the text.
levels correlate with CAT enzyme levels and the transcripts originate at 5' start sites known to be viral transcription start sites within the late promoter.

**Competition analysis.** To further demonstrate that the 21-bp repeat region (the rho element) is involved in the repression of the late promoter, competition experiments were performed. If interaction occurs between the 21-bp repeat region (rho element) and a putative repressor, then the addition of a competitor containing only the 21-bp repeat region (rho element) should compete for repressor binding, resulting in derepression of the active late promoter on the test CAT plasmid. Figure 4 shows that this is the case. In these experiments pL16-cat was added to the cells at a constant amount (5 μg/60-mm plate) with increasing amounts of competitor plasmids which contained (i) the 21-bp repeat region only, (ii) the entire late promoter, and (iii) the Rous sarcoma virus long terminal repeat. The data show that the addition of as little as 5 μg of the 21-bp repeat competitor causes the activity of the test promoter to increase, apparently due to the successful competition for a repressor. The addition of greater amounts of this competitor had no effect indicating that 5 μg constituted a saturating amount of competitor. By using the entire late promoter as a competitor we note that at low competitor levels the activity of the test promoter increases as it did with the 21-bp repeat (rho element) competitor; however, at higher concentrations activity falls off. We interpret this as indicating that the repressor can be successfully competed at a competitor concentration which has not affected the levels of transcription factors specifically required for late promoter activity. At the higher competitor concentrations the activity of the test promoter falls because of the titration of transcription factors which interact with other regions of the late pro-

**DISCUSSION**

The data presented above, as determined by deletion analysis and competition experiments, strongly suggest that the activity of the SV40 late promoter elements, tau and delta, does not occur de novo when T antigen mediates trans activation. Instead, the basal promoter activity of these elements appears to be repressed during the early phases of infection, before the appearance of a physiologically active concentration of T antigen. Once derepressed, this basal activity can be enhanced by the activation mechanisms mediated by T antigen. This would be a reasonable strategy for the viral lytic infection in which it is initially very important that transcription be dedicated to the early genes to establish a successful infection. The repression of the late promoter elements at early times may eliminate competition between the promoters so that the early promoter has the greatest advantage. Whether this effect relates to the multiple binding of the Sp1 transcription factor within the 21-bp repeat region (5) is not known at this point.

It can be argued that the increased activity seen when the 21-bp repeat region (rho element) is deleted results from inactivation of the early promoter thus freeing general transcription factors to interact with the late promoter. The competition data argue against this possibility. The specific increase in activity of the intact late promoter upon addition of increased amounts of rho element competitor, or low levels of the entire late promoter competitor, argues that a specific trans-acting factor is being competed or titrated. This result also indicates how the derepression may occur during the lytic infection. When a physiologically relevant level of T antigen is attained, the binding of T antigen at the viral origin of replication will allow replication. As the viral genome copy number increases, the limited supply of repressor will be titrated allowing the late promoter elements to become active at their basal level. Subsequently, further activation occurs due to the T-antigen-mediated mechanisms previously described (3, 7, 17). This derepression mechanism can explain a few previous data. It has been shown (24) that increased levels of template in an in vitro transcription reaction will result in detectable late promoter activity, whereas the late promoter is relatively inactive at lower template concentrations. In addition, Graessmann et al. (12) have demonstrated that late promoter activity can be detected after microinjection of template into the nuclei of CV-1 cells. In both of these cases relatively large amounts of template are either added to the in vitro reaction or injected into the nuclei. Thus, it is quite possible that under each circumstance a putative repressor is overcome by template number, resulting in derepressed basal promoter activity.

The possibility of a repressor of the late promoter which functions early in the lytic infection has also been suggested by previous data. Handa and Sharp (13) showed that late transcription could be increased early in the lytic cycle by infection in the presence of a protein synthesis inhibitor which presumably blocked the synthesis of the repressor. Ferdinand et al. (8) showed that transcriptional complexes isolated early in the lytic infection could be made to produce increased amounts of late transcripts by treating the complexes with Sarkosyl (CIBA-GEIGY Corp.); presumably this removed the repressor. In addition, Brady and Khoury
have suggested the possibility of late repression based on their competition studies of the SV40 late promoter. However, previous data (5, 14, 21) examining the late promoter by in vitro transcription analysis in HeLa cell extracts (hence under nonactivated conditions, but in a nonmonkey cell) suggest that the 21-bp repeat region is necessary for late promoter activity. At this point we can offer no explanation for the apparent discrepancy between the in vitro data and our in vivo transfection data, other than there being a fundamental difference between the two assay systems. For example, the in vitro system stresses utilization of the 5' end at SV40 nucleotide 170, whereas the in vivo analysis does not stress this site.

Based on a combination of the present data as well as our previous data examining the activity of the late promoter under T-antigen-activated conditions (18; see above), we can postulate a model for late promoter activity in vivo (Fig. 5). Upon entry of the viral DNA into the nucleus a factor binds within the 21-bp repeat region. This represses transcription in the late direction, but allows transcription to proceed in the early direction. We have designated this putative repressor region of the late promoter the rho (ρ) element. During a transition phase, T antigen reaches physiologically active concentrations and binds to the origin (omega) region. Based on the competition results discussed above, we postulate that viral genome amplification, resulting from T-antigen binding, may titrate the effect of the rho element-repressor interaction, thus initiating basal promoter activity and allowing T-antigen-mediated activation. Our previous data (18) suggest that T-antigen-mediated activation is the result of (i) activation of the omega-dependent delta element resulting from either the binding of T to the origin region or the act of replication and (ii) activation of the omega-independent tau element. As mentioned above, a variety of evidence suggests that the activation of the tau element may be mediated by a cellular trans-acting factor which is induced, activated, or modified by T antigen.

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LITERATURE CITED