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Cooperative Activation of Transcription by Bovine Papillomavirus Type 1 E2 Can Occur over a Large Distance

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The viral transcriptional factors encoded by the E2 open reading frame bind to the specific DNA sequence elements ACCGNNNNCGGT, allowing activation or repression of transcription. We have analyzed bovine papillomavirus type 1 E2 transactivation using recombinant genes containing E2-binding sites inserted at either 3′ or 5′ positions relative to the heterologous transcriptional initiation site of the herpes simplex virus thymidine kinase gene. In these hybrid plasmids, strong transactivation required the presence of a minimum of two E2-binding sites in close proximity to the promoter or five binding sites at a distance. The presence of a single E2-binding motif 5′, close to the initiation site, increased the efficiency of E2 transactivation from a distance in a more-than-additive manner. Since each E2-binding site bound a dimer of the E2 protein, these experiments suggest that transactivation by E2 requires the interaction between several E2 dimers with other essential transcription factors. This interaction may be facilitated by DNA looping, which would bring E2 molecules close to the promoter.

The regulation of transcription initiation in mammalian cells involves specific interactions between proteins and DNA sequences. These short DNA-binding sites of 10 to 20 base pairs are genetic elements which constitute the promoter and enhancer elements of the control regions of protein-coding genes (for a review, see reference 37). Transcription is usually initiated about 30 base pairs downstream from the TATA element of a promoter. Enhancer elements are defined by the ability to activate transcription regardless of their locations or orientations with respect to a promoter. The emerging concept pertaining to the regulation of transcription in mammalian cells is that despite these functional distinctions, enhancers and promoters are composed of similar elements with analogous roles in the control of transcription. The transcriptional modulator proteins bound to these sequences may interact with one another and with the basic transcriptional machinery in fundamentally the same way (6; P. Herbomel, Ph.D. thesis, University of Paris IV, Paris, France, 1989).

The products of the E2 open reading frame of the papillomaviruses constitute a family of DNA-binding transcriptional modulators (for a review, see reference 4). E2-binding sites are present several times in the genomes of all papillomaviruses sequenced so far, but their distribution and numbers vary among viral subtypes (3, 20; reviewed in R. Sousa, N. Dostatni, and M. Yaniv, Biochim. Biophys. Acta, in press). In bovine papillomavirus type 1 (BPV1) or in cottontail rabbit papillomavirus, binding of E2 to targets inside or just upstream of the major early promoter strongly activates transcription of viral early genes (9, 10, 31). In BPV1, transcription of the E2 gene is autoregulated via E2-binding targets located several kilobases upstream (16). In genital human papillomaviruses, E2-binding sites are located inside the viral promoter itself, very close to the TATA box (35). Finally, as in the case of the BPV1 P7185 promoter, an E2-binding site is found downstream from the transcription start site (34). The E2 proteins have been shown in these last two cases to repress the activity of the viral promoter (28a, 33, 36), probably because binding to their DNA targets interferes with the binding of cellular factors necessary for transcription (1, 2, 7, 33, 36).

The examples cited above indicate that the distance between the DNA-bound regulatory protein and the transcriptional start site can be crucial and may determine whether binding of the transcriptional regulatory factor will result in the activation or repression of transcription. Such a situation has been well described for the dual function of the lambda repressor (27). However, although repression of transcription can be easily explained by the capacity of the regulatory protein to bind DNA and sterically hinder the formation of the initiation complex, the mechanism of activation remains unknown. For E2, it has been clearly established that cooperation between at least two E2-binding motifs is required for efficient transactivation (30); two sites are sufficient for high E2-dependent activation when inserted about 100 nucleotides upstream of the transcriptional start site of heterologous promoters (11, 15). This effect was observed in different types of mammalian cells and was extended to yeast (18, 23, 32). Since E2 binds to its cognate sequence as a dimer (5, 21, 24), it appears that the functional E2 activator is composed of two dimers or a tetramer that presumably interacts with the other components of the transcriptional machinery.

One way to analyze the mechanism of activation by E2 is to compare the number of E2 DNA-binding targets required for efficient activation either in the vicinity of or at a distance from the transcriptional start site. For this purpose, various numbers of the E2-binding target were inserted either 5′ or 3′ or both to the chloramphenicol acetyltransferase (CAT) gene (13) driven by the herpes simplex virus thymidine kinase (TK) promoter. The promoter used in this study consists of 109 nucleotides upstream of the start site of the TK gene (22), including the TATA box, two binding sites for the transcription factor SP1 (17), and a CAATT box. The TK-CAT reporter plasmid used is depicted in Fig. 1. It contains a single *Xba*I restriction site at position −109

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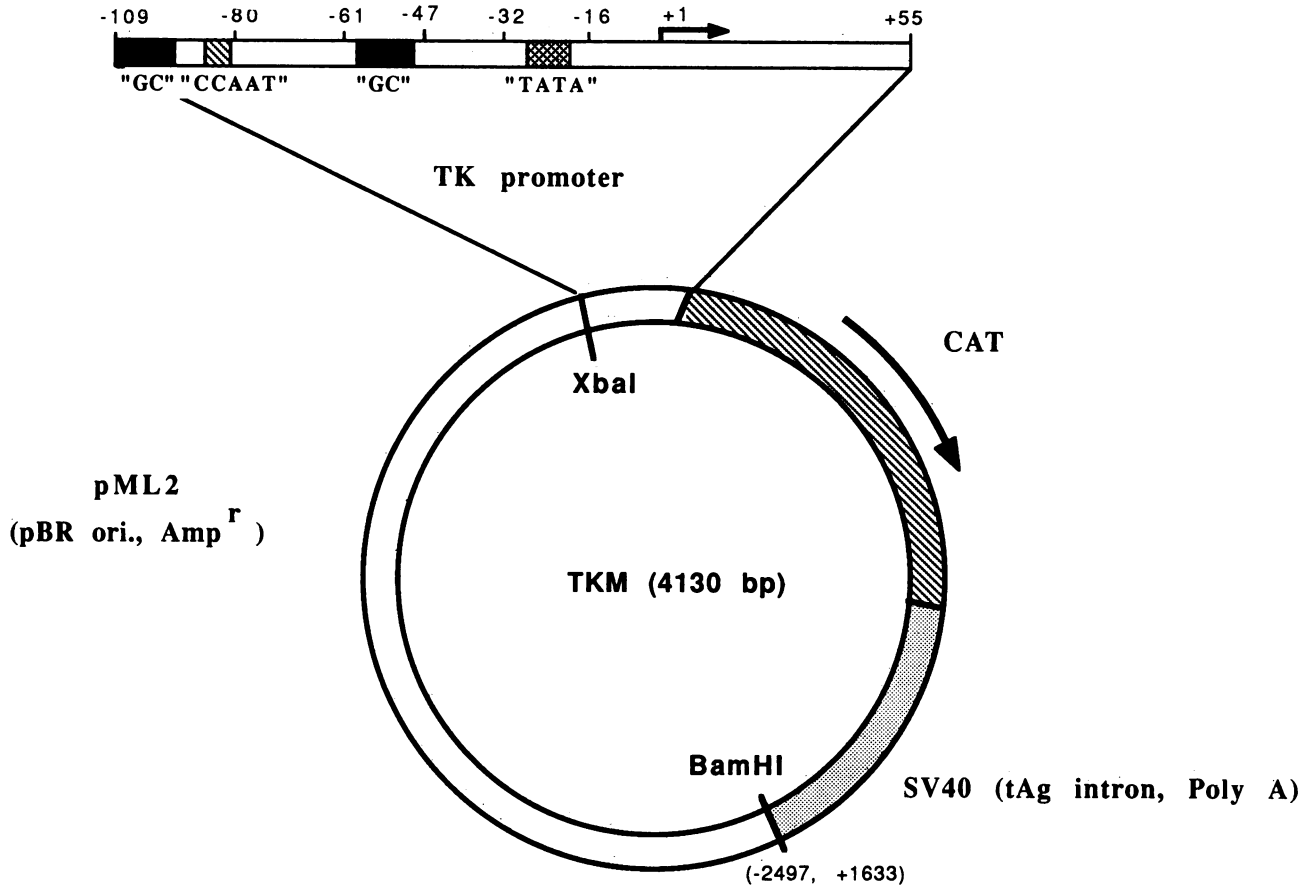


FIG. 1. Structure of the CAT reporter plasmids used in this study. The CAT reporter vector contains 109 nucleotides upstream and 55 nucleotides downstream of the transcription initiation site of the herpes simplex virus type 1 TK promoter, including the TATA box, a CCAAT box, and two GC boxes (binding sites for the SP1 transcription factor). The TK promoter drives the expression of the bacterial CAT-coding gene. The unique *Xba*I and *Bam*HI sites used for cloning of the E2-binding oligonucleotides at proximal and distal positions relative to the transcription start site are indicated. SV40, Simian virus 40; tAg, t antigen; bp, base pairs.

relative to the TK transcription start site and a unique *Bam*HI restriction site downstream of the CAT-coding sequences, 1.6 kilobase pairs from the TK initiation start site. Oligonucleotides containing one E2-binding site and cohesive *Xba*I or *Bam*HI ends were inserted in various numbers in the TK-CAT reporter plasmid (Fig. 2).

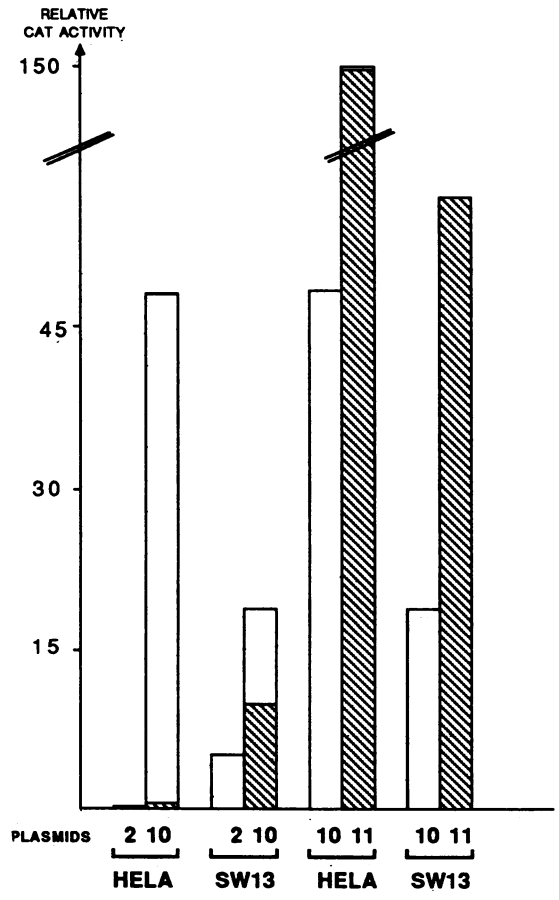
The reporter plasmids were cotransfected with the BPV-1 E2 expression plasmid pC59 (39). One microgram of the CAT target plasmids and different amounts of the E2 expression plasmid were introduced by the calcium phosphate coprecipitation method (38) into HeLa cells, a cervical carcinoma cell line containing human papillomavirus type 18 DNA sequences (29); C33 cells, a cervical carcinoma cell line known not to contain any human papillomavirus DNA sequences (40); and SW13 cells, an adrenocortical carcinoma cell line (19). To correct for variations in transfection efficiencies, all DNA samples also included the Rous sarcoma virus (RSV) β-galactosidase (12) or RSV luciferase (26) expression vectors as an internal control. Each transfection experiment was repeated at least twice, and the means of the normalized CAT values were calculated. All CAT plasmids showed the same average basal level in the absence of the E2 expression plasmid. This indicates that the E2-binding sites do not activate in the absence of the viral protein itself. Furthermore, although a recent study described a residual transactivation by E2 even when the CAT gene was driven by either viral or cellular promoters lacking the E2-binding target (14), we did not observe any such activation of the

FIG. 2. Transactivation by E2 of the different CAT plasmids. Structures of the plasmids used in this study. The number and orientations of the E2-binding sites are indicated by arrowheads, each corresponding to a single E2-binding palindrome ▶(ACCGAAAACGGT) flanked by an *Xba*I (for 5′ constructions) or *Bam*HI site (for 3′ constructions). CAT activities, given in arbitrary units, were normalized relative to the β-galactosidase or luciferase activities in SW13 cells or HeLa cells, respectively, and are the means of at least two experiments. The basal activity of the TK promoter (plasmid 1) was around 1 and 0.2% of that of the RSV long terminal repeat promoter in SW13 and HeLa cells, respectively, in the presence of E2. N.D., Not determined. (B) Synergy and additivity between proximal E2-binding sites in HeLa and SW13 cells. The relative CAT activity obtained in the presence of E2 of plasmids containing one or two (constructs 2 and 10) or two and six (constructs 10 and 11) binding sites is given. The activity expected from simple additivity in the action of sites for plasmids 10 and 11 is indicated (▨). (C) Synergy between proximal and distal sites. The degree of activation expected by simple additivity between 5′ and 3′ sites is indicated (▨).

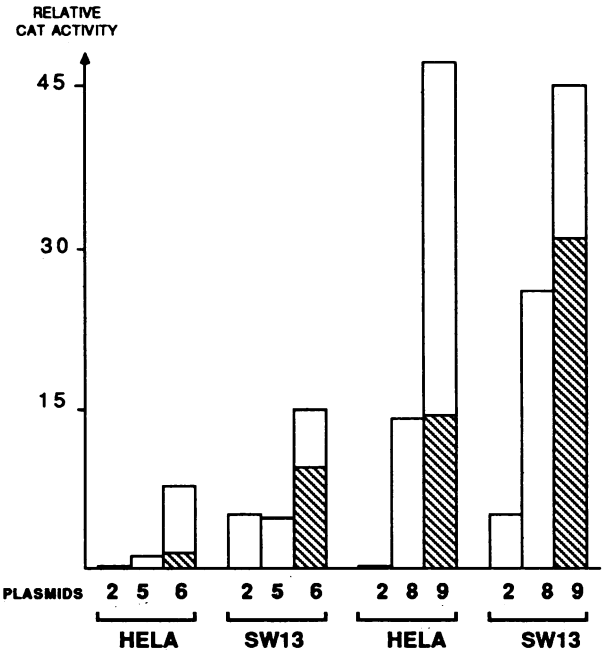
A)

CAT reporter plasmids: N°		Relative CAT activities in the presence of BPV1-E2 HELA	SW13
1	5' TK CAT 3'	1	1
2	TK CAT	1.3	6
3	TK CAT	1	2.1
4	TK CAT	1.6	11
5	TK CAT	2.3	5.7
6	TK CAT	8.7	15
7	TK CAT	N.D	6
8	TK CAT	15	27
9	TK CAT	48	44
10	TK CAT	50	20
11	TK CAT	150	56

B)



C)



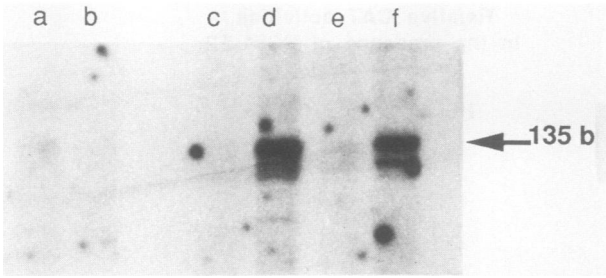


FIG. 3. Primer extension analysis. RNA was extracted as previously described (35) from C33 cells transfected by 2 μg of plasmid 1 (lane a), 2 μg of plasmid 1 with 1 μg of pC59 (lane b), 2 μg of plasmid 11 (lane c), 2 μg of plasmid 11 with 1 μg of pC59 (lane d), 2 μg of plasmid 2 with 1 μg of pC59 (lane e), or 2 μg of plasmid 9 with 1 μg of pC59 (lane f). CAT activities of 1/10 of the cell extract in a 1-h incubation period were, from lanes a to f, 1.2, 0.7, 0.4, 36, 3, and 40% conversion of chloramphenicol. Ten micrograms of total RNA was analyzed by primer extension with a labeled primer complementary to the 5′ region of the CAT gene (5′-TTGGGATATAT CAACGGTGG-3′). The expected size of the extended product to the TK cap site (22) was 135 nucleotides long. b, Bases.

RSV or TK promoters by E2 under our experimental conditions but did observe a weak inhibition of the TK promoter with high concentrations of the pC59 expression plasmid. However, in order to exclude any such effects, we analyzed the results obtained for each plasmid with identical concentrations of the E2 expression plasmid, the only variable parameter being the number and arrangement of E2-binding sites on the CAT reporter plasmids.

The presence of only one binding site inserted at the proximal position 5′ to the CAT gene had no effect on the expression of the TK promoter in HeLa cells but allowed a low but significant activation by E2 in SW13 (Fig. 2) or C33 (Fig. 3 and 4B) cells. This result indicates that a single dimer of E2 may activate transcription several fold in certain mammalian cells lines, as has been shown for yeast cells (18). As expected, a strong synergistic (i.e., more-than-additive) effect was observed between two sites inserted at the proximal position in both HeLa and SW13 cells (compare plasmids 2 and 10 in Fig. 2). Interestingly, when the number of E2 sites is further increased from two to six at the proximal position (plasmid 11), transcriptional efficiency increases in a linear fashion (compare plasmids 10 and 11 in Fig. 2). This suggests that two E2 dimers are the optimal transactivator. No major differences in activity were observed when the orientations of the palindromic E2-binding sites were reversed or when the distance between two palindromes varied from 5 to 25 base pairs (J. M. Gauthier and M. Yaniv, unpublished results).

In contrast to the situation described above, insertion of two E2-binding sites at the distal position 3′ to the CAT transcription unit (1.6 kilobases downstream or 3 kilobases upstream of the start site of the TK promoter, since this is a circular plasmid) allowed only a weak activation by E2. However, increasing the number of sites at the distal position from two to five caused a significant increase in activation of the TK promoter, an effect that is clearly more than additive (compare plasmids 5 and 8 in Fig. 2). These results show that the E2 protein is indeed able to activate transcription in mammalian cells at a distance when bound downstream of the initiation site but that transactivation by E2 is extremely sensitive to the distance of the E2 DNA targets from the initiation site. The difference in the number of sites required to achieve strong activation by E2 from either position suggests that two E2 dimers are unable to strongly activate transcription at a distance and need to cooperate with other 3′-bound E2 molecules. We confirmed by primer extension analysis that the CAT activations reported in this study were indeed due to an increase of the initiation of CAT mRNA transcription (Fig. 3). Furthermore, this experiment indicated that the same cap site was used with the TK-CAT plasmids regardless of the distal or proximal positions of the E2-binding sites.

The most intriguing result was that the presence of a single E2 site at the proximal position strongly increased the efficiency of transactivation by remote sites, although this single site by itself mediated only weak or no activation by E2. This synergy between the distal E2 sites and a single proximal E2 site was found for every number of distal sites tested. The activity of the different plasmids containing a single site at the proximal position and multiple sites at the distal position was always higher than the simple addition of both activities (compare plasmids 5 and 8 with plasmids 6 and 9, respectively, in Fig. 2A and C). To get some insight into the mechanism of this synergy, we varied the concentration of the E2 expression plasmid in the transfection experiments. Several plasmids containing different numbers of E2-binding targets showed roughly parallel increases in activity upon increases in the amount of transfected E2 expression plasmid (Fig. 4). The only exception was plasmid 2, containing only one E2 target at the proximal position, for which the plateau was reached with a very low amount (about 20 ng) of E2 expression plasmid (Fig. 4). In agreement with the results reported in Fig. 2, the maximal CAT activities were different for each plasmid. Comparison of panels A and B in Fig. 4 shows that the characteristics of E2 transactivation are comparable in the two different cell lines of SW13 and C33. Within the limit of sensitivity of this method, for all of the other plasmids that we tested, maximal E2 activation was reached with the same ratio of 0.1 μg of E2 expression plasmid for 1 μg of CAT plasmid. This indicates that the level of maximal activity depends only on the number of E2-binding targets in *cis*. By varying the amount of CAT reporter plasmids, we could show that the concentration of active E2 continues to rise with the increase in the quantity of E2-coding plasmid used (results not shown). It is plausible that occupancy of all of the E2-binding sites in the different plasmids will give various levels of intrinsic transactivation of the TK promoter, depending on the exact configuration of the different factors bound to the DNA molecule.

The results in Fig. 4 are compatible with a situation in which only a fraction of the test plasmid functions at its maximal rate when E2 is limiting. In other words, a limiting number of E2 dimers will not distribute randomly among the DNA templates but will bind cooperatively to only a fraction of the templates. This is surprising, since we did not observe strong cooperativity in the binding of E2 to DNA fragments containing several sites in vitro (18). At present, we can propose two possible explanations for this paradox. (i) Cooperativity in binding occurs in vivo, perhaps depending on labile posttranslational modifications of E2. (ii) Certain components of the transcriptional machinery stabilize the complex formed when several E2 dimers bind to the same template, perhaps by interacting with the N-terminal domain of the protein. We have to recall that similar observations were made with the yeast Gal4 protein. Although cooperativity between several sites occurs in vivo, no such effect was seen for DNA binding in vitro (8).

It is generally postulated that enhancers function at a

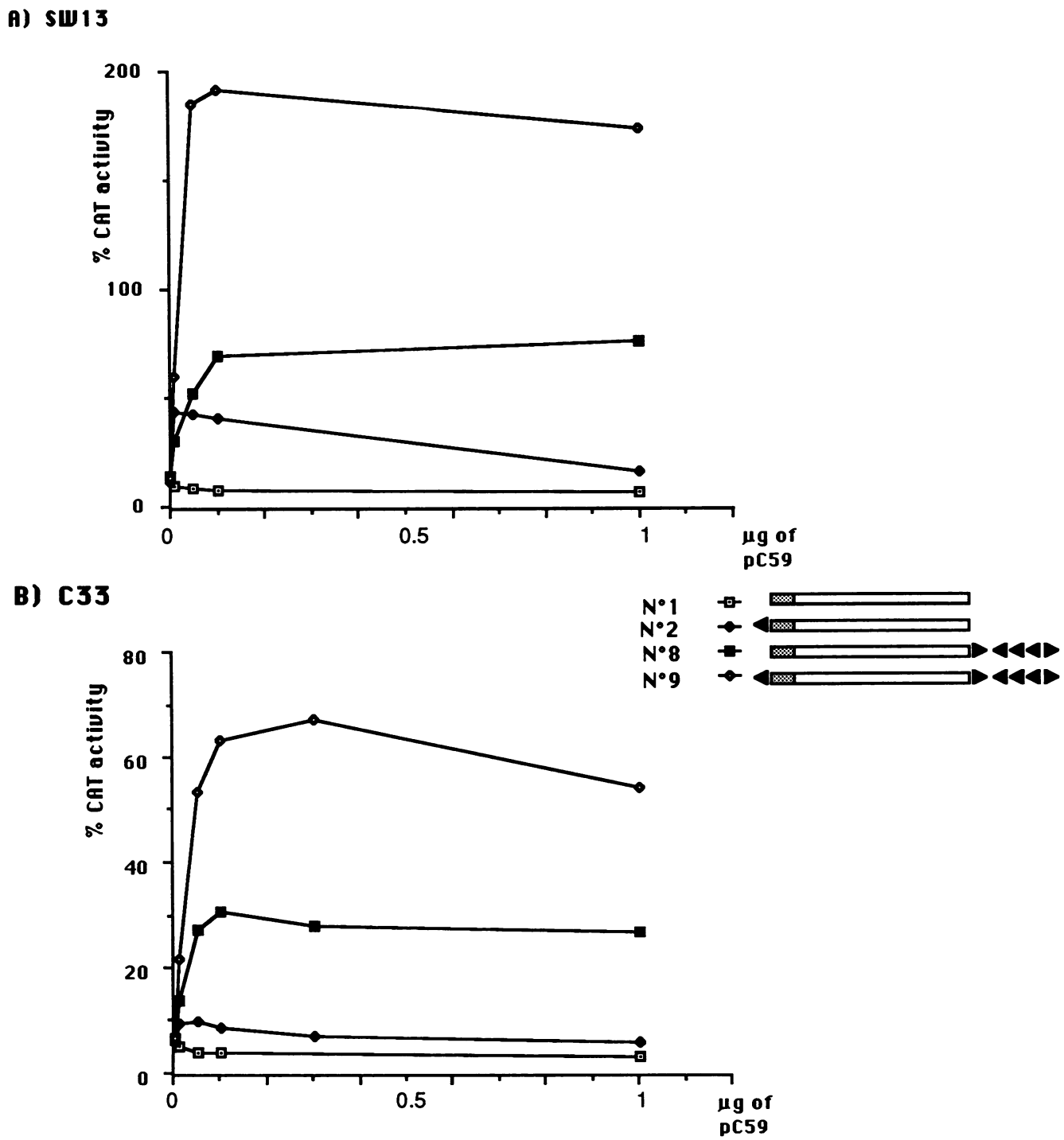


FIG. 4. Dose dependence of E2 activation for four different CAT plasmids. Cotransfection experiments were carried out with SW13 (A) and C33 (B) cells as described in the text with increasing concentrations of the BPV1 E2 expression plasmid pC59 and 1 µg of the different CAT-coding plasmids. One microgram of RSV β-Gal was used for each transfected plate, and the CAT activities were normalized relative to the β-Gal activity. The values plotted are the means of at least two independent series of transfection experiments. See the legend to Fig. 2A for an explanation of the plasmid structures.

distance by a mechanism that involves DNA looping (25, 28). Our results are compatible with such a model and contribute towards its understanding. We have shown that two E2 dimers (or a tetramer) have to bind close to the promoter in order to strongly activate transcription. This can occur by looping that will bring an E2 tetramer close to the promoter. In the absence of 5'-bound E2, the probability that looping brings E2 tetramers close to the promoter increases with the number of E2 sites present at the distal sites. The presence of an E2 dimer in a proximal position could increase the probability of forming a stable loop and a tetramer by E2-E2 contacts. E2 would then interact with a

transcription factor(s), such as SP1, CTF, or TFIID, bound in the vicinity of the transcription start site of the TK promoter.

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