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A mitigator sequence in the downstream region of the major late promoter of adenovirus type 12 DNA

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Human adenovirus type 12 (Ad12) replicates in permissive human host cells, but undergoes an abortive infection cycle in non-permissive hamster cells. Ad12 DNA cannot replicate and late viral genes are not expressed in hamster cells, whereas most of the early viral mRNAs are synthesized. We have shown previously that the major late promoter of Ad12 DNA (Ad12 MLP; nucleotides −228 to +435 relative to nucleotide +1 as the site of transcriptional initiation) does not function in uninfected or in Ad12-infected hamster BHK21 cells. The transcriptional defect of Ad12 DNA in hamster cells has thus been, at least partly, localized to the viral MLP. As expected, this construct is active in permissive human cells. Here, we show that the sequence between nucleotides +249 and +435 in the Ad12 MLP is in some way responsible for the late transcriptional block of this promoter in hamster cells. An Ad12 MLP–CAT construct comprising nucleotides −228 to +248 shows striking activity in hamster cells, and its activity is very markedly enhanced in Ad2- or Ad12-infected hamster or human cells compared with the nucleotide −228 to +435 construct. By using exonuclease *Bal*31, a series of Ad12 MLP–CAT gene assemblies were constructed which carry deletions of increasing lengths in the downstream part of the Ad12 MLP. Activity measurements of these constructs in BHK21 and in HeLa cells have located the presumptive mitigator element to the Ad12 sequence between nucleotides +320 and +352 of the MLP. It is also demonstrated that in the nucleotide −228 to +248 MLP construct, transcription is initiated at the authentic Ad12 MLP cap site after the transfection of both hamster and human cells. The localization of this cap site in the nucleotide sequence of the Ad12 MLP indicates the similarity to the comparable start site in the MLP of Ad2 DNA. The Ad2 MLP does not seem to harbour a comparable mitigator element. These results adduce evidence for the presence of a mitigator element in the first intron following the Ad12 MLP. This mitigator contributes to the abortive infection cycle to which Ad12 is subject in non-permissive hamster cells and might contribute to the relatively low efficiency in human cells.

Key words: species-specificity of viral promoter/non-permissivity of hamster cells for Ad12/Ad12 major late promoter defect/chloramphenicol acetyltransferase (CAT) assays/promoter–protein binding

Introduction

Viral systems offer ideal models for investigations on the species specificity of eukaryotic promoters, since viruses as mobile packaged genetic elements with long-standing biological experience can interact with hosts from different species. The non-permissivity of hamster cells for the replication of human adenovirus type 12 (Ad12) can, at least in part, be explained by the failure of the major late promoter (MLP) of Ad12 DNA to function in hamster cells. The same promoter is operational in human cells which are permissive for Ad12 replication. Thus, the abortive infection of hamster cells with Ad12 is due partly to the species specificity of this viral promoter (Weyer and Doerfler, 1985). In hamster cells, Ad12 DNA replication is not detectable (Doerfler, 1969; Fanning and Doerfler, 1976) and late viral genes are not transcribed, whereas early Ad12 genes are expressed (Raška and Strohl, 1972; Ortin and Doerfler, 1975. Ortin *et al.*, 1976; Esche *et al.*, 1979; Klimkait and Doerfler, 1985, 1987). In contrast, human adenovirus type 2 (Ad2) is capable of replicating in both human and in hamster cells (Strohl *et al.*, 1966; Doerfler, 1969).

During the productive infection of human cells by Ad2, the late genes of the L1 group are transcribed early in the infection cycle (Chow *et al.*, 1979; Akusjärvi and Persson, 1981; Nevins and Wilson, 1981). There is evidence that these genes are involved in the assembly of the adenovirion (Hasson *et al.*, 1989). The L1 segment of Ad12 DNA as well as Ad12 VA (virus associated) RNA fail to be transcribed in hamster cells (Jüttermann *et al.*, 1989). We have shown previously that the E1 functions of Ad2 or of adenovirus type 5 (Ad5), in particular genes encoded in the viral E1B region, can complement the defect of Ad12 DNA replication and late gene transcription in hamster cells (Klimkait and Doerfler, 1985, 1987). However, in spite of Ad12 DNA and late RNA being synthesized under the complementing influence of Ad2 or Ad5 E1 functions, Ad12 late proteins and virions do not seem to be produced in appreciable amounts. Hence, in addition to the aforementioned Ad12 deficiencies in hamster cells, there must be hitherto unidentified functions which are lacking for the assembly of infectious Ad12 virions (Klimkait and Doerfler, 1985, 1987).

In the present report, it is shown that in the DNA segment between nucleotides +320 and +352, relative to the site of initiation of transcription in the MLP of Ad12 DNA, there resides a sequence element that inactivates this promoter in hamster cells and reduces its activity in human cells. This mitigator element may be responsible for the failure of the Ad12 MLP to function in hamster cells. By using the band shift (migration delay) technique in electrophoresis experiments on polyacrylamide gels, the binding of specific proteins from hamster or human cells to the mitigator

element has been detectable. The functional relationship of these proteins to promoter inactivation in hamster cells has not yet been clarified.

Results

Species dependence of the major late promoter of Ad12 DNA

It has been documented that the MLP of Ad12 DNA with the prokaryotic chloramphenicol acetyltransferase (CAT) gene as reporter does not function in uninfected or Ad12-infected BHK21 cells, but that the defective Ad12 promoter could be transactivated in Ad2-infected BHK21 cells (Weyer and Doerfler, 1985). In that sense, the MLP of Ad12 DNA reflected the deficiency of the Ad12 viral genome in non-permissive hamster cells. It seemed likely that there were several defects in the Ad12 genome to account for the abortive cycle of Ad12 in hamster cells. In previously published analyses, these defects had been localized to the E1B region (Klimkait and Doerfler, 1987), to the VA RNA region (Jüttermann *et al.*, 1989), and to the MLP of Ad12 DNA (Weyer and Doerfler, 1985). It was conceivable that these functions were somehow interdependent since all were subject to E1A regulation. There was evidence that in Ad12-infected BHK21 cells, at least one of the E1A-encoded mRNAs, i.e. that responsible for the synthesis of a 34 kd protein, failed to be available in hamster cells (Esche *et al.*, 1979). On the other hand, the possibility existed that the species-specific defect of the MLP of Ad12 DNA was attributable to a sequence within that promoter.

We, therefore started to prepare MLP constructs of Ad12 and Ad2 DNA which encompassed different segments of each of these promoters and carried the CAT gene as indicator. The maps in Figure 1 outline the details of these constructions. All map coordinates are presented as nucleotide numbers upstream (− = minus) or downstream (+ = plus) from nucleotide +1, which is the site of transcription initiation in the MLPs (arrows in Figure 1). The nucleotide sequence of the Ad12 MLP (−228/+248) fragment was redetermined in the new construct and found to be identical to previously published data (Shu *et al.*, 1986). These DNA constructs were transfected as circularized plasmids into uninfected or Ad2- or Ad12-infected human HeLa or hamster BHK21 cells; the levels of CAT activity in cellular extracts were determined at 30−48 h after transfection by standard techniques.

Transcriptional initiation in the MLP of Ad12 DNA

The site of initiation of transcription in the MLP of Ad12 DNA has not previously been determined. Therefore cytoplasmic RNA was isolated from HeLa cells at 24 h after infection (p.i.) with Ad12. The isolated RNA was hybridized to the ^{32}P-labelled −228/+248 DNA fragment of the MLP of Ad12 DNA. The DNA−RNA hybrids were then treated with S1 nuclease. The data of Figure 2a demonstrate that two major fragments of 252 and 248 nucleotides and two minor fragments of 204 and 202 nucleotides were protected from S1 cleavage. Thus, there were four potential, perhaps alternative, sites for the initiation of transcription in the MLP of Ad12 DNA (Figure 2b). Comparison of the Ad2 and Ad12 nucleotide sequences in the cap site regions of the MLP revealed that the site designated as +1 in the MLP of Ad12

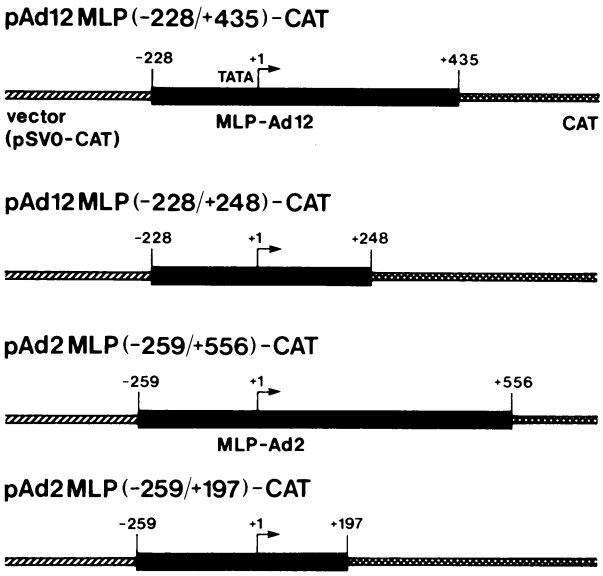


Fig. 1. Four different Ad12 and Ad2 MLP constructs containing the chloramphenicol acetyltransferase (CAT) gene. Details of the construction are as described in Materials and methods. The circular map was linearized for presentation. The site of transcription initiation (cap site) in the MLP sequences is designated +1 and by a bent arrow. The numbers indicate nucleotide positions relative to the +1 cap site. The location of the TATA signal in the promoter is also shown.

(Figure 2b) corresponds to the cap site in the MLP of Ad2 DNA. The latter cap site was determined previously by Evans *et al.* (1977) and Ziff and Evans (1978).

A downstream sequence in the MLP of Ad12 is responsible for its deficiency in hamster cells

Initially the activity of the MLP constructs was tested in uninfected HeLa and BHK21 cells. The data presented in Table I summarize the outcome of several independent experiments. It is apparent that the pAd12MLP(−228/+435)-CAT construct had a markedly lower activity, if any at all, in hamster BHK21 cells than the pAd12MLP(−228/+248)-CAT construct which lacked the +249/+435 MLP segment. CAT activity in the pSV2-CAT DNA was governed by the early SV40 promoter and served as a positive control. The plasmid pSV0-CAT, which lacked a eukaryotic promoter, was also used as a control but had low activity levels both in hamster and in human cells. The pSV0-CAT activity was probably due to the transactivation of plasmid nucleotide sequences by cellular proteins as demonstrated previously (Langner *et al.*, 1986). The pAd12MLP(−228/+248)-CAT DNA also showed higher activity in human cells than the construct with the longer Ad12 MLP element [pAd12MLP(−228/+435)-CAT]. There was some variation in the absolute levels of CAT activity, as was frequently observed with this test system for promoter functionality. The data indicate that a negative regulatory element, located downstream in the Ad12 MLP between nucleotides +249 and +435, was responsible for the failure of this MLP to function in hamster cells (Weyer and Doerfler, 1985; this report).

It was also found that the Ad2 MLP constructs, which carried promoter sequences between (−259/+556) or between (−259/+197), both had low activities in hamster and in human cells (Table I). There were no apparent

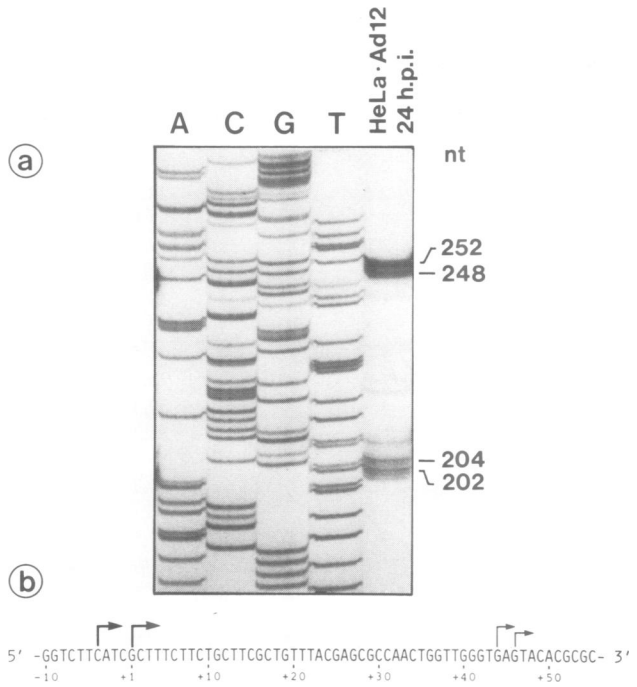


Fig. 2. S1 protection analysis of RNA from Ad12-infected HeLa cells and mapping of the sites of transcription initiation in the MLP of Ad12 DNA. (**a**) Some of the experimental details are described in the text. The hybridization probe was labelled 5'-terminally at nucleotide +248 in the following way: the pAd12MLP(−228/+435)-pBR322 construct was cleaved with *BglII* at position +248. The linearized fragment was labelled using polynucleotide kinase and [γ-^{32}P]ATP. The plasmid was then recut with *Eco*RI at position −228 and the labelled fragment was purified by electrophoresis on a 4% polyacrylamide gel to produce a 5' end-labelled DNA fragment (see map in Figure 4). An amount of 5' labelled DNA corresponding to 10 000 c.p.m. (Cerenkov) was annealed at 42°C to 5 μg of total cytoplasmic RNA (isolated 24 h p.i. of HeLa cells with Ad12) in a volume of 30 μl. The DNA−RNA hybrids were treated for 1 h at 30°C with 300 units of S1 nuclease per 300 μl reaction mixture (Weisshaar *et al.*, 1988). The diagnostic ^{32}P-labelled DNA fragments were resolved on a 6% polyacrylamide gel in 7 M urea (Maxam and Gilbert, 1980). As size markers, the sequence reaction products for A, C, G and T of M13mp18 DNA were co-electrophoresed. The dideoxy method (Sanger *et al.*, 1977) was employed and the 17mer universal M13 primer (Pharmacia) was used. The gels were autoradiographed on Kodak XAR-5 film. (**b**) The nucleotide sequence (Shu *et al.*, 1986) of part of the Ad12 MLP is shown, indicating the four potential transcriptional initiation sites.

differences in the expression levels of the Ad2 MLP constructs comparable to those for the Ad12 MLP constructs. Thus no inhibitory element in the Ad2 MLP was detectable in uninfected cells.

In the actual adenoviral infection cycle, the viral MLP was transactivated by other viral and possibly by virus-dependent cellular functions. Hence, the activity of the MLP constructs in hamster and human cells had also to be assessed in Ad2- or Ad12-infected hamster and in human cells.

Inhibitory function of a downstream sequence in the Ad12 MLP in adenovirus-infected hamster or human cells

The results of several independent experiments are summarized in Table II. These data confirm and extend the conclusions of the previous section. The infection of both hamster and human cells with Ad2 or Ad12 led to higher activity levels of the promoter−CAT gene constructs studied. Both in hamster and in human cells, the pAd12MLP(−228/+248)-CAT construct, which was devoid of the inhibitory sequence between nucleotides +249 and +435 had a strikingly higher activity than the pAd12MLP(−228/+435)-CAT construct after infection of the cells with Ad2 or Ad12. For the Ad2 MLP construct which lacked the +198/+556 sequence, there was only a modest increase of the activity in Ad2-infected hamster or human cells, if any at all, in comparison with the pAd2MLP(−259/+556)-CAT construct.

The activity of the Ad12 MLP could best be judged in Ad12-infected cells, since it was known that the MLP required other viral and/or virus-dependent cellular functions for full activity. The pAd12MLP(−228/+435)-CAT construct was inactive in uninfected and in Ad12-infected BHK21 cells, but had some activity in Ad2-infected BHK21 cells due to transactivation (Weyer and Doerfler, 1985). The pAd12MLP(−228/+435)-CAT construct also had low activity in Ad2- or in Ad12-infected HeLa cells. Upon removal of the +249/+435 segment from the Ad12 MLP, it acquired considerable activity in uninfected BHK21 cells (Table I) and markedly higher activity levels in Ad2- or in Ad12-infected BHK21 cells (Table II). It was concluded that the downstream +249/+435 segment of the Ad12 MLP was responsible for the lack of this promoter's activity both in uninfected and in Ad12-infected hamster BHK21 cells.

Table I. Increased activity of the pAd12MLP(−228/+248)-CAT construct in uninfected BHK21 and HeLa cells

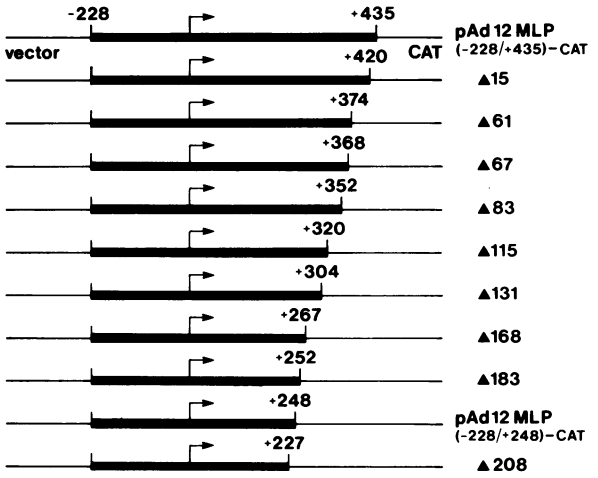
Cells	pSV0-CAT	pSV2-CAT	pAd2MLP (−259/+556)-CAT	pAd2MLP (−259/+197)-CAT	pAd12MLP (−228/+435)-CAT	pAd12MLP (−228/+248)-CAT
BHK21	8.1	69.2	14	19	1.7	52
	15.9	86.2	2.8	7.1	1.4	41.3
	10.5	87	2.1	1.9	0.9	5.9
	4.7	88.6	4.5	2.1	0.7	4.3
	7	91.6	6.7	2.9	0.6	5.7
HeLa	19	97	1.9	2.3	1.4	6.8
	5.8	94.3	2.8	1.6	1.2	5.7
	11.1	93.7	7.7	3	1.2	5

The conversion of ^{14}C-labelled chloramphenicol (CAM) to acetylated forms of CAM by CAT was determined in extracts of cells which were transfected with the constructs indicated. Standard methods for CAT assays were employed (Gorman *et al.*, 1982; Kruczek and Doerfler, 1983; Gorman, 1985). After the resolution of CAM from its acetylated derivatives by TLC, the ^{14}C-labelled compounds were localized on the chromatogram by autoradiography, the carrier plus compound corresponding to individual spots were cut out, and the radioactivity in individual spots was measured in a scintillation counter. The data represent percentage values of the acetylated forms of CAM relative to the amount of total CAM. The results of several independent experiments are included.

Table II. Increased activity of the mitigator-free MLP promoter of Ad12 DNA in Ad2- or Ad12-infected BHK21 or HeLa cells

Cells	pSV0-CAT	pSV2-CAT	pAd2MLP (−259/+556)-CAT	pAd2MLP (−259/+197)-CAT	pAd12MLP (−228/+435)-CAT	pAd12MLP (−228/+248)-CAT
BHK21						
Ad2-infected	2.9	53.5	8.1	18	2.9	62
	3.9	94.6	7.7	23.4	2.5	38.7
	6.9	93.6	16.7	23.8	4.2	83.8
	3.6	92.5	1.5	7.3	3.8	49.7
BHK21	72.8	89.4	13.2	3.3	1.7	30
Ad12-infected	28.5	59.3	4	7.7	0.8	27
	9.5	69.1	8.5	8.8	1.6	42.5
	16	52.2	10.1	7.1	1	26.7
	35.4	90.4	38.8	14.1	1.1	39.3
	4.7	89.3	3.8	3.7	0.5	35.3
HeLa	2.4	4.6	7	20.8	1.3	59.7
Ad2-infected	0.7	9	6	2.4	0.4	36.1
	55.5	68.3	1	12	3.1	47.5
HeLa	3.8	96.8	7.9	29.8	7.6	87.1
Ad12-infected	0.9	91.4	11.6	11.9	5.2	37.5
	5.4	41.7	18.8	10.9	3.5	47.1

Experimental details are described in the text and were similar to those outlined in the legend to Table I, except that infected cells were transfected with the plasmid constructs shown. Again, the results of several independent experiments are presented.



BHK21					HeLa		
uninf.		Ad12 inf.			uninf.	Ad12 inf.	
1	1	0.9	0.7	0.5	0.5	22.7	7.6
2	0.7	1	0.8	0.3	0.3	10.8	3.6
1	0.3	0.4	0.5	0.26	0.2	2.7	3.4
1	0.3	0.8	0.6	0.4	0.3	29.4	10.3
1.2	0.3	0.3	0.5	0.2	0.2	4.9	1.9
25.6	3.5	3.9	1.3	2.4	1.9	91.5	64.4
49.7	n.d.	15.1	4.7	n.d.	1.4	70.6	n.d.
52	25.8	n.d.	1.2	6.3	1.2	n.d.	93.2
81.2	15.1	n.d.	14.4	6.2	n.d.	n.d.	60.8
9.6	17	9.4	10.3	3	1.5	93.9	67.6
28.9	9.1	n.d.	3.2	4.3	n.d.	n.d.	32.9

Fig. 3. Promoter lengths and biological activities of various Ad12 MLP constructs in uninfected or in Ad12-infected BHK21 or HeLa cells. The construction of the Ad12 MLP−CAT gene assemblies with exonuclease *Bal*31 was detailed in Materials and methods. The circular map was linearized for ease of presentation. The vector, MLP (heavy line), and CAT parts of the constructs are indicated, together with the site of transcription initiation (cap site, bent arrows). Nucleotide numbers are relative to the cap site (+1). The lengths of the promoter deletions (Δ) in individual constructs are designated in nucleotides deleted at the downstream end, relative to the −228/+435 promoter fragment. Uninfected or Ad12-infected BHK21 or HeLa cells were transfected with each of the Ad12 MLP−CAT gene constructs which carried deletions of increasing lengths as indicated. At 44−48 h after transfection, extracts were prepared, and the conversion of ^{14}C-labelled chloramphenicol (CAM) to acetylated forms of CAM by CAT activity in extracts of cells transfected with the described constructs was determined. Experimental details were similar to those described in the footnotes to Tables I and II. The results of several independent experiments are reported as the percentage of CAM converted to acetylated forms of CAM.

Precise localization of the inhibitory element in the downstream region of the Ad12 MLP

The exact position of the inhibitory element in the downstream segment of the Ad12 MLP was determined by constructing a series of Ad12 MLP−CAT gene assemblies which carried deletions of increasing lengths in the downstream area of the Ad12 MLP. The activities of these constructs were then tested in uninfected or in Ad12-infected BHK21 or HeLa cells. The structures of the assemblies used in these experiments are schematically characterized and the measured activities summarized in Figure 3. The data demonstrate that the inhibitory sequence element was most probably situated between nucleotides +320 and +352. Both in uninfected or in Ad12-infected BHK21 or HeLa cells, a

dramatic increase in the activities of the Ad12 MLP−CAT gene assemblies was observed between construct Δ 83, which had no activity in BHK21 and low activity in HeLa cells, and construct Δ 115 which showed significant activity in BHK21 and markedly increased activity in HeLa cells. Removal of the segment between nucleotides +304 and +320 (Δ 131) might lead to a further activity increase of the construct in BHK21 cells. However, the precision of the CAT assay system did not in that case permit a definite conclusion to be made. It is concluded that the nucleotide sequence between +320 and +352, relative to the cap site, in the downstream segment of the Ad12 MLP harbours an element that abolishes promoter function in hamster cells and that mitigates activity in human cells.

Transcription initiation at the authentic cap site in the modified Ad12 MLP containing the −228/+248 segment

Was the transcriptional activity of the shorter Ad12 MLP fragment initiated at the authentic cap site of this promoter? To answer this question the total cytoplasmic RNA from uninfected, Ad2- or Ad12-infected BHK21 or HeLa cells, which had been transfected with the pAd12MLP(−228/+435)-CAT or the pAd12MLP(−228/+248)-CAT construct, was isolated 30−48 h after transfection and analysed by the standard S1 protection procedure used in our laboratory (Weisshaar *et al.*, 1988). As illustrated in the map in Figure 4, the −228/+248 DNA fragment of the Ad12 MLP was used as hybridization probe; this probe was ^{32}P-labelled and purified as described in the legend to Figure 2.

Upon infection of HeLa cells with Ad12, RNA was synthesized which protected nucleotide fragments 252/248 and 204/202 in S1 protection experiments (Figure 4, lanes a−c). The same 252/248 nucleotide fragments were protected by RNA isolated from Ad2-infected BHK21 cells which were transfected with the pAd12MLP(−228/+435)-CAT construct (cf. map in Figure 1; Figure 4, lane e), whereas in uninfected or in Ad12-infected BHK21 hamster cells this RNA was apparently not produced (Figure 4, lanes d and f). Transfection of Ad2-infected BHK21 cells with the pAd12MLP(−228/+248)-CAT construct, which lacked the mitigator element, also led to the synthesis of RNA which protected a 254 nucleotide fragment (Figure 4, lane h). The slightly increased length of this fragment was due to the presence of a *Hin*dIII linker on the probe. Similar results were obtained with RNA from Ad12-infected BHK21 cells (Figure 4, lane i), although the signal was weaker than with RNA from Ad2-infected cells. The mitigator-less construct was not expressed in uninfected BHK21 cells (Figure 4, lane g). Yeast tRNA did not protect the specific promoter fragment of Ad12 DNA (Figure 4, lane j; Figure 5, lane i).

Infection of HeLa cells with Ad12 virions again led to the synthesis of cytoplasmic RNA which protected the same 252/248 and 204/202 nucleotide fragments from the MLP of Ad12 DNA (Figure 5, lanes a and b) as RNA from Ad2- or Ad12-infected HeLa cells which had been transfected with the pAd12MLP(−228/+435)-CAT (Figure 5, lanes d and e) or the pAd12MLP(−228/+248)-CAT construct (Figure 5, lanes g and h). In the latter experiment, again a 254 nucleotide fragment was protected. The RNA from uninfected HeLa cells did not yield a signal when the construct containing the mitigator was transfected (Figure 5, lane c) or a very weak signal when the mitigator-less construct was used (Figure 5, lane f).

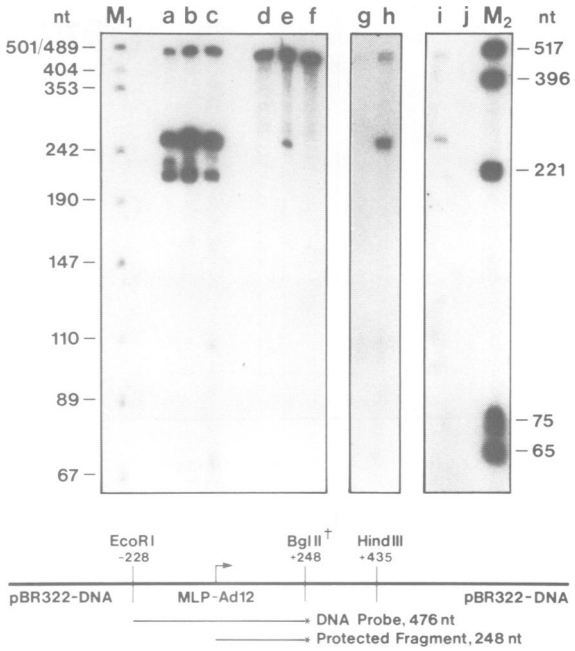


Fig. 4. Transcription of the pAd12MLP(−228/+248)-CAT construct in BHK21 cells was initiated at the authentic cap sites of the Ad12 MLP. Experimental conditions are described in the text. The graph presents an autoradiogram of S1 nuclease-protected DNA fragments separated by electrophoresis on 5% polyacrylamide gels in 7 M urea. The map location in the Ad12 MLP and the site of labelling (*) of the 476 nucleotide DNA probe, which was used for the protection hybridization with RNA preparations (see below) in S1 nuclease protection experiments, are shown. The protected 248 nucleotide fragment is also indicated in the map. The symbol † at the *Bgl*II restriction site indicates the modification of this site by a *Hin*dIII linker to facilitate ligation into the same site of the pSV0-CAT plasmid. Results of different experiments are shown by spaces between the parts of the autoradiogram. The lengths of the protected fragments can be compared by reference to the standard fragment length markers used: (**M$_1$**) pUC18 plasmid DNA was cut with *Hpa*II, (**M$_2$**) pUC18 DNA was cut with *Hin*fI and the fragments were ^{32}P-labelled at their 5′ termini by polynucleotide kinase and [γ-^{32}P]ATP. The RNA preparations employed for S1 protection experiments were derived from the following sources. **Lanes a−c**, HeLa cells were infected with Ad12, and the RNA was isolated at 23 (**a**), 32 (**b**) and 24 h (**c**) p.i. The results of three independent experiments using 5, 4 and 8 μg of RNA, respectively, are shown. **Lanes d**, uninfected (66 μg RNA), (**e**) Ad2-infected (25 μg RNA), or (**f**) Ad12-infected BHK21 cells (58 μg RNA) transfected with the pAd12MLP(−228/+435)-CAT plasmid which contained the mitigator. **Lanes g**, uninfected (32 μg RNA), (**h**) Ad2-infected (18 μg RNA) or (**i**) Ad12-infected BHK21 cells (29 μg RNA) transfected with the mitigator-free pAd12MLP(−228/+248)-CAT construct. Time intervals after infection and/or transfection are described in Materials and methods. **Lane j**, yeast tRNA (20 μg) was used for hybridization experiments and afforded no protection. Other experimental conditions are described in the legend to Figure 2. Nucleotide lengths (nt) of marker fragments are indicated.

The described nucleotide lengths of the protected DNA fragments (Figures 4 and 5) demonstrate that the authentic start sites in the Ad12 MLP in Ad12-infected HeLa cells were also used in Ad2- or in Ad12-infected BHK21 or HeLa cells after transfection with the pAd12MLP(−228/+435)-CAT construct or the mitigator-less pAd12MLP(−228/+248)-CAT construct in front of the CAT gene. In

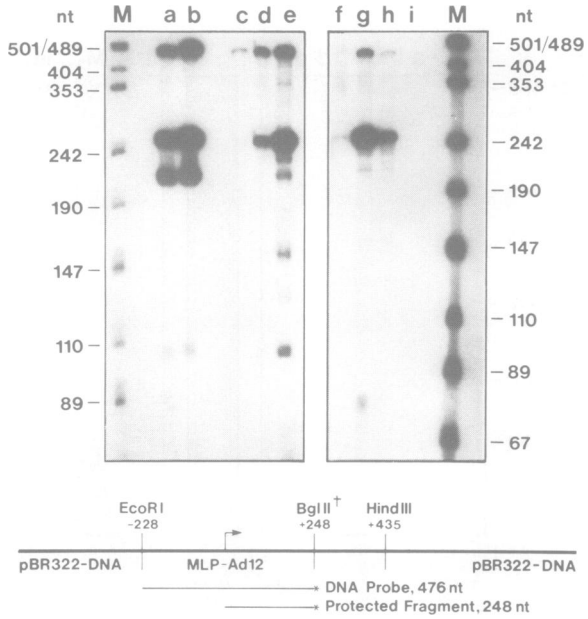


Fig. 5. Mapping of the sites of transcription initiation in the MLP of Ad12 DNA in infected and transfected HeLa cells. The experimental details were similar to those described in the legend to Figure 4. The RNA preparations for the protection of the ^{32}P-labelled DNA probe (cf. map) against cleavage by S1 nuclease were derived from the following sources: **lanes a,b** Ad12-infected HeLa cells; RNA (5 μg, 4 μg) was isolated at 23 and 32 h p.i., respectively. **Lanes c**, uninfected (17 μg RNA), (**d**) Ad2-infected (10 μg RNA) or (**e**) Ad12-infected HeLa cells (11 μg RNA) transfected with the pAd12MLP(−228/+435)-CAT plasmid. **Lanes f**, uninfected (13 μg RNA), (**g**) Ad2-infected (8 μg RNA) or (**h**) Ad12-infected HeLa cells (8 μg RNA) transfected with the pAd12 MLP(−228/+248)-CAT plasmid. **Lane i**, yeast tRNA (20 μg RNA). The marker lanes (**M**) contained the 5′ γ-^{32}P-labelled *Hpa*II fragments of pUC18 DNA and their lengths are indicated in nt. The autoradiogram is of a dried 5% polyacrylamide gel in 7 M urea.

uninfected BHK21 cells, the Ad12 MLP constructs did not seem to be expressed (Figure 4, lanes d and g).

It is concluded that the pAd12MLP(−228/+248) promoter construct was recognized at the authentic start sites, which were used in permissive cells and in the entire, non-manipulated viral genome. This construct was also transcribed in BHK21 cells which behaved non-permissively *vis-à-vis* the pAd12MLP(−228/+435) promoter fragment. Apparently, the +249/+435 segment of the Ad12 MLP carried an inhibitor which exerted its effect in the milieu of non-permissive hamster BHK21 cells and also of permissive human HeLa cells.

Binding of proteins to the downstream element in the Ad12 MLP

The possibility existed that the inhibitory effect of the downstream Ad12 MLP fragment in hamster cells would be reflected in an altered capacity of that fragment to bind to proteins from uninfected Ad12- or Ad2-infected hamster or human cells. Nuclear extracts from uninfected (Figure 6a), from Ad12-infected (Figure 6b) or from Ad2-infected (Figure 6c) BHK21 or HeLa cells were compared for the presence of protein components that could bind differentially to the downstream Ad12 MLP fragment between nucleotides +320 and +352 (Figure 6). Experimental details of the preparation of nuclear extracts, the binding reaction and the analytical band shift assays are described in Materials and methods or in the legend to Figure 6. The results obtained from migration delay experiments on 4% polyacrylamide gels, summarized in Figure 6, did not demonstrate significant differences in the binding patterns of proteins (complexes 1 to 3) from the nuclear extracts prepared from uninfected Ad12- or Ad2-infected BHK21 or HeLa cells (Figure 6a−c). Upon very close inspection, there might be minute differences among the more slowly migrating nucleoprotein complexes (complex 1). However, we could not assign functional significance to these observations. When synthetic oligodeoxyribonucleotides were used which comprised nucleotides +304 to +352 or +320 to +368 in the downstream region of the Ad12 MLP (see Figure 7), band shift data were obtained which were very similar to those presented in Figure 6.

There were also minute differences in the binding patterns of nuclear proteins from BHK21 and HeLa cells to the much longer (+216/+435) nucleotide fragment from the downstream region of the Ad12 MLP (data not shown). However, it could not be ascertained in what way these binding differences were responsible for the inhibitory function that this downstream element bestowed upon the Ad12 MLP.

Discussion

Eukaryotic promoters as important genetic elements are able to respond to an array of environmental conditions. With viral promoters it is possible to assess their potency in cells of different species. Such studies may contribute to an improved understanding of factors that co-determine the species specificity of viral promoters and in that way may influence the host range of a virus. These factors will have to be sought among cellular proteins which possess or lack the capacity to interact with specific genetic elements in viral promoters. We have analysed the abortive infection of hamster cells with human Ad12 in considerable detail. One of the motivations for this investigation has been the oncogenic potential of Ad12 in newborn hamsters (Trentin *et al.*, 1962; Kuhlmann *et al.*, 1982).

It has been demonstrated that the MLP of Ad12 is unable to function in hamster cells, even when they have been infected by Ad12 and when most of the early Ad12 functions are provided in the nucleus of the infected cells (Weyer and Doerfler, 1985). In contrast, Ad2 infection of hamster cells, which are permissive for Ad2, transactivates the MLP of Ad12 in hamster cells when this promoter is introduced jointly with a reporter gene into Ad2-infected cells. Under these conditions, the MLP of Ad12 is rendered accessible to the activation by viral and cellular factors. Of course, in human cells the MLP of Ad12 can be shown to be functional and to activate also the CAT gene which has been used as the genetic indicator in this series of experiments. At least part of the deficiency in the expression of the Ad12 genome in hamster cells can be attributed to the species specificity of the MLP of Ad12 DNA.

The results described in this report adduce evidence for the notion that a mitigator element or an inhibitory sequence in the downstream region of Ad12 DNA is accountable for its inability to function in BHK21 hamster cells. By using the CAT indicator gene as a reporter, it has been

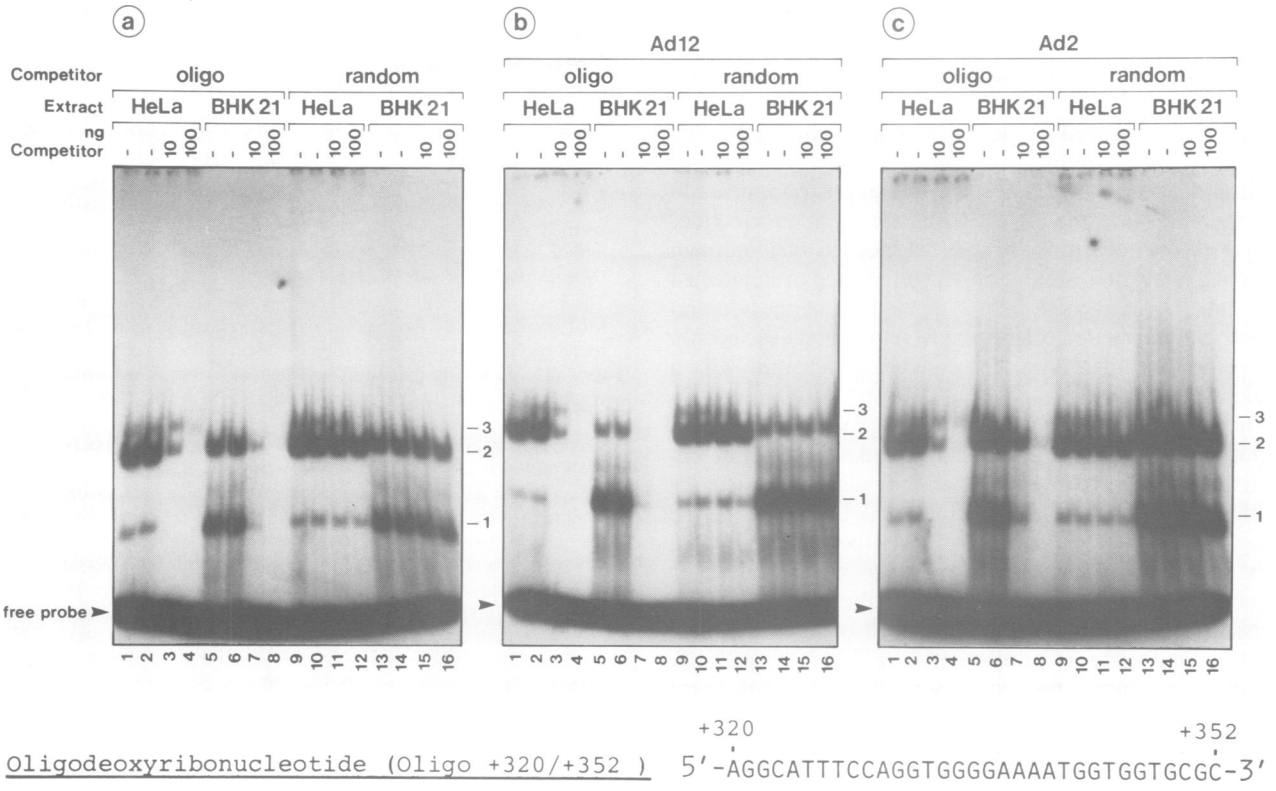


Fig.6. Protein binding to the mitigator element: band shift analyses with nuclear extracts from uninfected or adenovirus-infected hamster or human cells. Band shift experiments were performed as described in Materials and methods. The 5′ end-labelled oligodeoxyribonucleotide used is shown below the gels. As unspecific competitor, 1.5 μg of poly(dA-dT) alternating co-polymer was added to each reaction. In some of the experiments, 10 or 100 ng of the unlabelled oligodeoxyribonucleotide was included in the reaction mixture as specific competitor. An oligodeoxyribonucleotide of random sequence (cf. Materials and methods) served as unspecific competitor. The positions of the uncomplexed, ^{32}P-labelled oligodeoxyribonucleotide in the gels are marked by arrows. The retarded nucleoprotein complexes are marked 1, 2 and 3. (**a**) Nuclear extracts of uninfected cells, either 2 μg of HeLa cell extract or 4 μg of BHK21 cell extract. (**b**) Two micrograms of Ad12-infected HeLa cell extract prepared 21 h p.i. or 4 μg of Ad12-infected BHK21 cell extract prepared 20 h p.i. (**c**) Two micrograms of Ad2-infected HeLa cell extract prepared 16 h p.i. or 4 μg of Ad2-infected BHK21 cell extract prepared 16 h p.i. Lanes 1 and 2, 5 and 6, 9 and 10, and 13 and 14 refer to double determinations in the absence of competitor.

demonstrated that the removal of a sequence located between nucleotides +320 and +352, i.e., of 33 nucleotides in the first intron of the late viral mRNAs of Ad12, bestows activity upon the MLP of Ad12 DNA even in hamster cells. The presumptive intron function of this nucleotide sequence has been derived from the corresponding sequence in Ad2 DNA and has not yet been directly documented for Ad12 DNA. Removal of the mitigator element also enhances the MLP activity of Ad12 DNA in permissive human cells (Tables I and II). A similar element has not been found in the MLP of Ad2 DNA. Nevertheless, a comparison between Ad12 and Ad2 DNAs of the nucleotide sequences in the MLP downstream region does not reveal major differences between the two viral DNAs (Figure 7). It is worth noting that the two contiguous 3-nucleotide exchanges between Ad12 DNA and Ad2 DNA reside within the nucleotide segment +320 to +352 (brackets in Figure 7) which harbours the mitigator element. Band shift experiments using the mitigator element as a synthetic oligodeoxyribonucleotide and nuclear extracts from uninfected Ad12- or Ad2-infected hamster or human cells have not revealed significant differences between the two species (Figure 6). In these experiments, the formation of nucleoprotein complexes 1 and 2 with nuclear extracts from both uninfected and infected HeLa or BHK21 cells is not a non-specific event. The

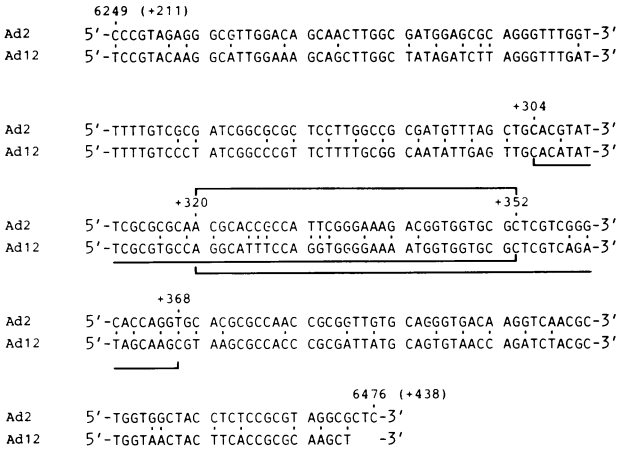


Fig. 7. Comparison of the nucleotide sequences in the downstream regions of the MLP in the Ad2 and Ad12 genomes. These nucleotide sequences were determined for Ad2 DNA by Roberts *et al.* (1986) and for Ad12 DNA by Shu *et al.* (1986). The numbers 6249 and 6476 refer to the nucleotide sequence of Ad2 DNA; +211, +304, +320, +352, +368 and +438 indicate nucleotide positions in the MLP of Ad2 relative to position +1 of the cap site. Differences between the Ad2 and Ad12 sequences are designated by a vertical line between nucleotides. The brackets indicate the oligodeoxyribonucleotides that were used to determine DNA–protein interactions by the band shift method.

appearance of these complexes can be prevented by adding the unlabelled oligodeoxyribonucleotide as specific competitor to the reaction mixture. In contrast, the addition of a synthetic oligodeoxyribonucleotide of 50 bp with a computer-randomized sequence does not compete for the binding of nucleoprotein complexes 1 and 2. Hence, formation of the two DNA−protein complexes appears to be specific for the 33 bp oligodeoxyribonucleotide. However, the occurrence of the less specific nucleoprotein complex 3, which is present only in uninfected or in infected HeLa nuclear extracts, can be competed out by the addition of both the unlabelled oligodeoxyribonucleotide as specific competitor and the 50 bp oligodeoxyribonucleotide with a randomized sequence.

The results described led to the formulation of the mitigator hypothesis. This genetic element of unknown origin, but clearly of viral provenance, might alleviate the efficiency of the major late Ad12 promoter and thus lead to the mitigation of viral pathogenicity in the permissive human host. At the same time, the presence of the mitigator sequence restricts viral expression in the non-permissive hamster cells to the realization of most of the early viral functions and to the integration pathway. The latter pathway can entail oncogenic transformation which is a rare event in the Ad12-hamster cell system, probably because, for transformation to occur, the expression schedule of several cellular functions has to be reoriented according to an unknown, complicated mode which might be influenced by Ad12 infection and viral DNA integration (Doerfler, 1968, 1970). Could the mitigator element facilitate a less destructive interaction of Ad12 with its host and save some of the host cells for future viral generations?

Materials and methods

Cells, virus and virus infections

Human HeLa or hamster BHK21 cells were grown in monolayer cultures in Dulbecco medium (Bablanian *et al.*, 1965) supplemented with 10% fetal bovine serum. For adenovirus propagation, HeLa cells were maintained in suspension cultures. Human Ad2 or Ad12 were produced and purified as described previously (Doerfler, 1969). Viral DNA was extracted from CsCl purified virions by the SDS−proteinase K-phenol method (Doerfler *et al.*, 1972). For transfection experiments (see below), HeLa cells in monolayer culture (25 cm^2 surface area, 60 mm diameter Falcon dishes) were inoculated with Ad2 or Ad12 at 20−25 plaque forming units (p.f.u.) per cell. BHK21 cells were inoculated at 80−100 p.f.u. of Ad12 per cell or with 30−50 p.f.u. of Ad2 per cell. At 16−18 h p.i., cells were transfected with promoter−indicator gene constructs as described below.

DNA constructs

For all tests assessing the activity of promoter constructs, the prokaryotic chloramphenicol acetyltransferase (CAT) gene was used as reporter. All constructs were generated starting with the original pSV0-CAT plasmid (Gorman *et al.*, 1982; Gorman, 1985). The following promoter constructs (schemes given in Figure 1) were prepared employing standard restriction and ligation protocols using *Hin*dIII linkers to anchor promoter fragments in the *Hin*dIII site of the pSV0-CAT plasmid. The pAd12MLP(−228/+435)-CAT construct contained the MLP fragment of Ad12 DNA comprising the designated nucleotides relative to the major cap site of the Ad12 MLP. All nucleotide numbers in the MLP of Ad12 DNA were calculated relative to this site. The pAd2MLP(−259/+556)-CAT plasmid carried the corresponding MLP fragment of Ad2 DNA in the *Hin*dIII site of the pSV0-CAT construct. These two promoter−CAT gene constructs were described and used in a previous study (Weyer and Doerfler, 1985). In a similar way, the pAd12MLP(−228/+248)-CAT and pAd2MLP(−259/+197)-CAT constructs were made which both contained shorter MLP fragments lacking 187 and 359 nucleotides, respectively, in comparison with the original MLP−CAT gene constructs.

For some of the experiments, Ad12 MLP constructs were prepared in which increasing lengths of the downstream region of the promoter were deleted. The pBR322 plasmid pAd12MLP(−228/+435) was cleaved with *Hin*dIII and incubated for 1−5 min with *Bal*31 exonuclease at 30°C. Subsequently, the DNA was cut with *Eco*RI, and the staggered termini were filled-in using the four deoxyribonucleotide triphosphates and Klenow polymerase (Klenow *et al.*, 1971). The DNA fragments were fitted with *Hin*dIII linkers and ligated into the *Hin*dIII site of the plasmid pSV0-CAT (Gorman *et al.*, 1982). Figure 3 shows the exact structure of each of the *Bal*31-generated and recloned constructs. Each recloned construct was purified and resequenced (see below).

Determination of nucleotide sequences

The pAd12MLP(−228/+248)-CAT promoter construct and all the Ad12 MLP constructs with *Bal*31-generated deletions (Δ in Figure 3) were partly resequenced to ascertain the promoter coordinates in these constructs. In these experiments, the dideoxy chain-termination method was applied (Sanger *et al.*, 1977) using α-[^{35}S]deoxyribonucleoside triphosphates.

Transfection of cells with plasmid constructs and assay for CAT activity

Uninfected or Ad2- or Ad12-infected human HeLa or hamster BHK21 cells were transfected at 16−18 h p.i. with promoter−CAT gene constructs by the calcium phosphate precipitation technique (Graham and van der Eb, 1973). At 4 h or, in deviation from established protocols, as late as 22 h after the addition of the DNA−Ca^{2+} precipitates, the cultures were treated for 2 min with 15% glycerol in HEPES-buffered saline. Subsequently, the cells were washed, fresh medium was added, and incubation was continued at 37°C. Extracts of transfected, uninfected or Ad12-infected cells were prepared 44−48 h after transfection and extracts of Ad2-infected cells at 30−48 h after transfection. CAT activity was determined according to standard procedures (Gorman *et al.*, 1982; Kruczek and Doerfler, 1983; Gorman 1985).

Other methods of molecular biology

Southern blotting (Southern, 1975) RNA transfer experiments (Northern blotting) (Alwine *et al.*, 1977; Lehrach *et al.*, 1977), preparation of cytoplasmic RNA (Scott *et al.*, 1983), the 5′ labelling of DNA fragments by polynucleotide kinase and [γ-^{32}P]ATP (Richardson, 1965), and S1 nuclease protection experiments (Berk and Sharp, 1977; Weisshaar *et al.*, 1988) were performed following published protocols.

Preparation of nuclear extracts from HeLa or BHK21 cells

Published procedures (Dignam *et al.*, 1983) were adapted. HeLa cells were grown in suspension cultures to a density of 4×10^5−7×10^5 cells/ml. BHK21 cells were propagated to subconfluency in monolayer cultures. In some experiments, HeLa or BHK21 cells were infected with Ad2 or Ad12 and harvested at 16−21 h after infection. The cells were washed twice at 0−4°C with phosphate-buffered saline deficient in Mg^{2+} and Ca^{2+} (Dulbecco and Vogt, 1954) and subsequently resuspended in 5 vol of lysis buffer [10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulphonic acid), pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT)] for 10−20 min at 0°C. The cells were pelleted again and resuspended in 2 vol of this buffer. The cytoplasmic membranes were ruptured by 10−15 (HeLa cells) or 25−30 (BHK21 cells) strokes in a tight fitting Dounce homogenizer. The liberated nuclei were resuspended in 3 ml of 20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethyl sulphonic acid (PMSF), 2.5 units of aprotinin per ml, 25% glycerol for 10^9 nuclei and were stirred on ice for 30−60 min. The nuclear extract was freed of debris by centrifuging at 14 500 r.p.m. for 25 min at 0°C (Beckman JA-20). The supernatant was either dialysed directly against 20 mM HEPES, pH 7.9, 20 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM each of DTT and PMSF, 2.5 units of aprotinin per ml, 20% glycerol or after precipitating proteins in the supernatant with 52% $(NH_4)_2SO_4$ (4°C, pH 8.0) and resuspension in the dialysis buffer. The dialysate (6 h, three changes of buffer) was clarified by centrifugation, frozen in liquid N_2 and stored at −80°C,

Synthetic oligodeoxyribonucleotides

Oligodeoxyribonucleotides of predetermined sequence compositions were synthesized in an Applied Biosystems 381A DNA synthesizer using the phosphoramidites of individual deoxyribonucleosides for the reactions.

Assay of DNA−protein complexes by the band shift method

Published procedures (Fried and Crothers, 1981; Garner and Revzin, 1981; Carthew *et al.*, 1985) were adapted. The synthetic oligodeoxyribonucleotides to be tested for their interactions with proteins were 5′-terminally labelled using [γ-^{32}P]ATP and polynucleotide kinase. An amount of DNA

corresponding to ~20 000 c.p.m. (Cerenkov) was incubated at 25°C for 30 min with 1.5 μg of sonified poly(dA-dT) alternating copolymer (Pharmacia) as unspecific competitor and 2–4 μg of protein from nuclear extracts in a total volume of 20 μl of 4 mM Tris–HCl, 12 mM HEPES, pH 7.9, 80 mM KCl, 5 mM $MgCl_2$, 0.6 mM each of EDTA and DTT, and 12% glycerol. DNA–protein complexes and the unbound DNA fragment were resolved by electrophoresis at ambient temperature on 4% polyacrylamide gels (acrylamide:bis-acrylamide = 38:2) in 50 mM Tris–HCl, 38 mM sodium-borate, 12.5 mM EDTA. After electrophoresis, the gels were dried on Whatman 3MM paper and autoradiographed at −80°C on Kodak XAR-5 film.

For competition experiments, 10 or 100 ng of the unlabelled, double-stranded oligodeoxyribonucleotide was added to the reaction mixture prior to the addition of the extracts. Usually the unlabelled competitor had the same sequence as the ^{32}P-labelled oligodeoxyribonucleotide (cf. Figure 6). In some of the competition experiments, a 50 bp synthetic oligodeoxyribonucleotide of random sequence was used as unspecific competitor. In this competitor, the four deoxyribonucleotides were represented in the proportions A:C:G:T = 13:13:10:14. The random sequence (5′-TGACTAGCATGTACCGCGTATTAATTCCCCAAACCTTGCA-AGGAGCTTTG-3′) was devised according to the method of Grob and Stüber (1987).

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References

Akusjärvi,G. and Persson,H. (1981) *Nature*, **292**, 420–426.
Alwine,J.C., Kemp,D.J. and Stark,G.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5350–5354.
Bablanian,R., Eggers,H.J. and Tamm,I. (1965) *Virology*, **26**, 100–113.
Berk,A.J. and Sharp,P.A. (1977) *Cell*, **12**, 721–732.
Carthew,R.W., Chodosh,L.A. and Sharp,P.A. (1985) *Cell*, **43**, 439–448.
Chow,L.T., Broker,T.R. and Lewis,J.B. (1979) *J. Mol. Biol.*, **134**, 265–303.
Dignam,J.D., Lebovitz,R.M. and Roeder,R.G. (1983) *Nucleic Acids Res.*, **11**, 1475–1489.
Doerfler,W. (1968) *Proc. Natl. Acad. Sci. USA*, **60**, 636–643.
Doerfler,W. (1969) *Virology*, **38**, 587–606.
Doerfler,W. (1970) *J. Virol.*, **6**, 652–666.
Doerfler,W., Lundholm,U. and Hirsch-Kauffmann,M. (1972) *J. Virol.*, **9**, 297–308.
Dulbecco,R. and Vogt,M. (1954) *J. Exp. Med.*, **99**, 167–182.
Esche,H., Schilling,R. and Doerfler,W. (1979) *J. Virol.*, **30**, 21–31.
Evans,R.M., Fraser,N., Ziff,E., Weber,J., Wilson,M. and Darnell,J.E. (1977) *Cell*, **12**, 733–739.
Fanning,E. and Doerfler,W. (1976) *J. Virol.*, **20**, 373–383.
Fried,M. and Crothers,D.M. (1981) *Nucleic Acids Res.*, **9**, 6505–6525.
Garner,M.M. and Revzin,A. (1981) *Nucleic Acids Res.*, **9**, 3047–3060.
Gorman,C. (1985) In Glover,D.M. (ed), *DNA Cloning*, vol. II, IRL Press, Oxford, pp. 143–190.
Gorman,C.M., Moffat,L.F. and Howard,B.H. (1982) *Mol. Cell. Biol.*, **2**, 1044–1051.
Graham,F.L. and van der Eb,A.J. (1973) *Virology*, **54**, 536–539.
Grob,U. and Stüber,K. (1987) *CABIOS*, **3**, 243–244.
Hasson,T.B., Soloway,P.D., Ornelles,D.A., Doerfler,W. and Shenk,T. (1989) *J. Virol.*, **63**, 3612–3621.
Jüttermann,R., Weyer,U. and Doerfler,W. (1989) *J. Virol.*, **63**, 3535–3540.
Klenow,H., Overgaard-Hansen,K. and Patkar,S.A. (1971) *Eur. J. Biochem.*, **22**, 371–381.
Klimkait,T. and Doerfler,W. (1985) *J. Virol.*, **55**, 466–474.
Klimkait,T. and Doerfler,W. (1987) *Virology*, **161**, 109–120.
Kruczek,I. and Doerfler,W. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 7586–7590.
Kuhlmann,I., Achten,S, Rudolph,R. and Doerfler,W. (1982) *EMBO J.*, **1**, 79–86.
Langner,K.-D., Weyer,U. and Doerfler,W. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 1598–1602.
Lehrach,H., Diamond,D., Wotney,J.M. and Boedtker,H. (1977) *Biochemistry*, **16**, 4743–4751.
Maxam,A.M. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499–560.
Nevins,J.R. and Wilson,M.C. (1981) *Nature*, **290**, 113–118.
Ortin,J. and Doerfler,W. (1975) *J. Virol.*, **15**, 27–35.
Ortin,J., Scheidtmann,K.-H., Greenberg,R., Westphal,M. and Doerfler,W. (1976) *J. Virol.*, **20**, 355–372.
Raška,K.,Jr. and Strohl,W.A. (1972) *Virology*, **47**, 734–742.
Richardson,C.C. (1965) *Proc. Natl. Acad. Sci. USA*, **54**, 158–165.
Roberts,R.J., Akusjärvi,G., Aleström,P., Gelinas,R.E., Gingeras,T.R., Sciaky,D. and Pettersson,U. (1986) In Doerfler,W. (ed.), *Developments in Molecular Virology*, vol. 8, Martinus Nijhoff Publishing, Boston, pp. 1–51.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Scott,M.R.D., Westphal,K.-H. and Rigby,P.W.J. (1983) *Cell*, **34**, 557–567.
Shu,L., Hong,J.S., Wei,Y. and Engler,J.A. (1986) *Gene*, **46**, 187–195.
Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503–517.
Strohl,W.A., Rouse,H.C. and Schlesinger,R.W. (1966) *Virology*, **28**, 645–658.
Trentin,J.J., Yabe,Y. and Taylor,G. (1962) *Science*, **137**, 835–841.
Weisshaar,B., Langner,K.-D., Jüttermann,R., Müller,U., Zock,C., Klimkait,T. and Doerfler,W. (1988) *J. Mol. Biol*, **202**, 255–270.
Weyer,U. and Doerfler,W. (1985) *EMBO J.*, **4**, 3015–3019.
Ziff,E.B. and Evans,R.M. (1978) *Cell*, **15**, 1463–1475.

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