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Properties of the Adenovirus Type 40 E1B Promoter That Contribute to Its Low Transcriptional Activity

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The adenovirus type 5 (Ad5) E1B promoter contains two elements essential for maximal activity, a TATA box and a GC box. The enteric adenovirus type 40 (Ad40) E1B promoter has a TATA box sequence identical to that of Ad5 and a GC box that fits the Sp1 binding site consensus. Nevertheless, Ad40 E1B RNA synthesis is severely impaired in HeLa cells, attributable in part at least to the weak transactivating activity of Ad40 E1A. However, the responsiveness of Ad40 early promoters to E1A transactivation has not been directly demonstrated. Using a transient expression assay with a chloramphenicol acetyl transferase (CAT) reporter gene, the Ad40 E1B promoter was very poorly transactivated by E1A of both Ad40 and Ad5 and showed only a limited response to the promiscuous varicella zoster virus transactivator p140. Construction of Ad5 recombinant viruses expressing the CAT gene under the control of the Ad5 or Ad40 E1B promoter allowed detection and measurement of expression from the Ad40 E1B promoter in a well-defined background and showed that overall activity is some 100-fold lower than for the Ad5 E1B promoter. Deletion analysis revealed that sequences upstream of the Sp1 binding site down-modulated Ad40 E1B promoter responsiveness, and two protein binding sites, identified by DNase footprinting and gel retardation assay, may be implicated in this effect. Gel shift analysis also showed that the Ad40 Sp1 binding site had a reduced affinity for Sp1 protein, relative to the Ad5 site, and that the context as well as the core sequence had an influence on Sp1 recognition.

Key Words: adenovirus; E1B; transcription; promoter.

INTRODUCTION

Growth restriction in tissue culture is a distinctive feature of the enteric adenovirus types 40 and 41 (reviewed by Mautner *et al.,* 1995; Tiemessen and Kidd, 1995). The E1 region has been implicated in this growth defect since both serotypes, although they grow poorly in HeLa cells, can be propagated in 293 cells (Takiff *et al.,* 1981), which express the Ad5 E1A and E1B genes (Graham *et al.,* 1977). Failure of Ad40 to grow in cells expressing Ad2E1A alone and efficient growth in cells expressing only the Ad2 E1B region, as well as in cells with the whole of the Ad2 E1 region (Mautner *et al.,* 1989), indicate the involvement of the E1B region in this phenotype. By comparing the synthesis of Ad40 E1B mRNA under the control of either the Ad40 or the Ad5 E1B promoter in an Ad5 virus background, we found that the overall level is reduced 8-fold in permissive 293 cells and 80-fold in nonpermissive HeLa cells (Bailey *et al.,* 1994). In addition, there were differences in the processing of the E1B transcripts in the different cell types, making it difficult to ascribe all of the differences exclusively to E1B promoter function. Because the E1B proteins have functions that affect the outcome of an infectious cycle in a number of different ways, we decided to make plasmid and virus constructs with the E1B coding region substituted by the chloramphenicol acetyl transferase gene, in order to measure more directly the activity of the E1B promoter.

For Ad2 and Ad5, the E1B promoter was originally described as a simple structure consisting of a TATA box and an Sp1 binding site (Wu *et al.,* 1987). Basal activity in the absence of E1A can be demonstrated for the TATA box alone, and this is augmented 5-fold when the Sp1 site is in its usual position 11 bases upstream of the TATA box. E1A transactivation of the TATA box or the TATA box plus the GC box increases the activity another 10-fold. E1A activation of E1B is mediated through the TATA box—only mutations in the TATA box affect E1A transactivation (Wu *et al.,* 1987). Upstream sequences also have a positive regulatory function, stimulating transcription some 20-fold, and binding sites for various transcription factors have been identified in this region (Parks *et al.,* 1988; Spector *et al.,* 1993).

The minimal Ad40 E1B promoter is similar in sequence to E1B promoters of other serotypes (Bailey *et al.,* 1994): It has a TATA box identical to that of Ad5 and a potential Sp1 binding site nine bases upstream (Fig. 1). Thus differences in E1B gene expression between Ad40

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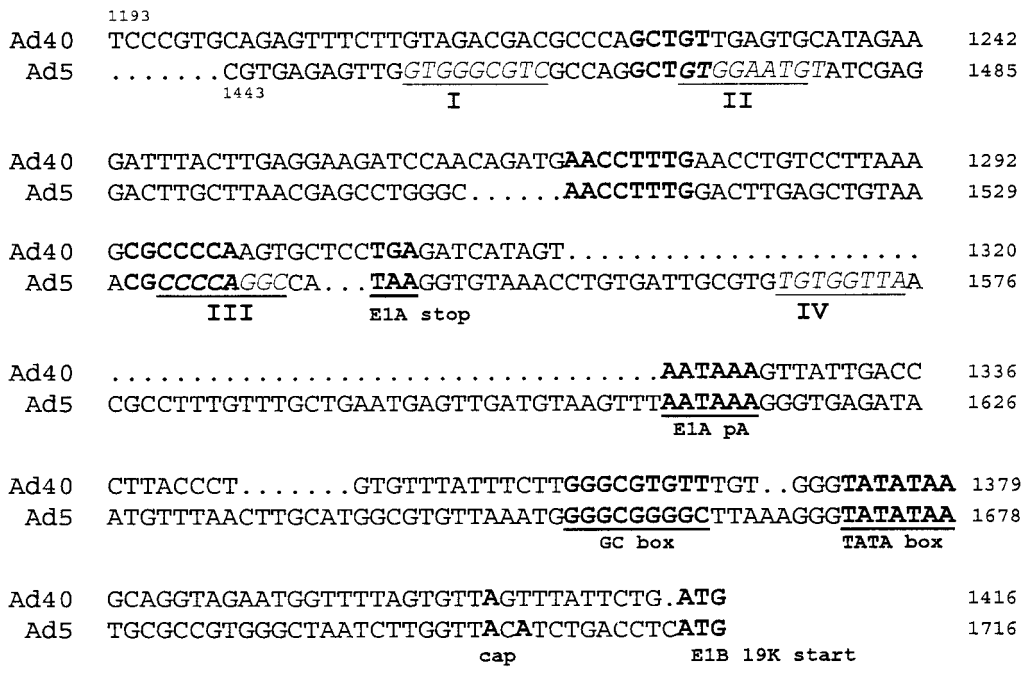


FIG. 1. Comparison of the E1B promoter and upstream regions of Ad5 and Ad40. The common features are in boldface type, and the regions of Ad5 designated boxes I–IV are in italics. Sequence numbering from GenBank M73260 (Ad5) and L19443 (Ad40).

and Ad5 do not reflect obvious differences in sequence of the major promoter elements. However, the upstream region, which overlaps with the end of the E1A coding region and 3′ signals for E1A mRNA processing, does differ considerably between Ad40 and Ad5, as shown in Fig. 1. The Ad40 intergenic region is much smaller than that for Ad5, and the putative transcription factor recognition signals identified for Ad5 (Parks *et al.,* 1988; Spector *et al.,* 1993) are not conserved. The experiments described here were designed to explore the differences between the Ad5 and the Ad40 E1B promoters, particularly with regard to the utilization of the GC box and the possible role of upstream sequences in transcription regulation. The contribution of upstream sequences was revealed by use of a set of virus constructs bearing progressive deletions in the E1A–E1B intergenic region. The importance of the GC box (Sp1 binding site) to the activity of the promoter was examined in gel shift assays, using homologous and heterologous competitors to assess the relative roles of the GC box and flanking sequences.

RESULTS

E1B promoter constructs

Four plasmids were constructed that contain the Ad40 E1B TATA box and mRNA cap site adjacent to the coding sequences for the chloramphenicol acetyl transferase (CAT) gene, with incremental amounts of upstream Ad40 sequence (Figs. 1 and 2A). The shortest construct (pVS9CAT, nt 1368–1411) includes only the TATA box, the second (pVS10CAT, nt 1352–1411) extends just beyond the putative Sp1 binding site, the third (pVS11CAT, nt 1298–1411) extends beyond the E1A poly(A) signal, and the longest (pVS12CAT, nt 1057–1411) is comparable in length to the Ad5 sequence in pE1BCAT, extending into the E1A intron.

E1B promoter activity in plasmid constructs

When the full-length Ad40 promoter construct (pVS12CAT) was introduced into HeLa cells by transfection using DOTMA, its basal activity was lower than that of pE1BCAT (Fig. 3A) and when cotransfected with a plasmid expressing a transactivator the Ad40 promoter was also less active than pE1BCAT (Steinthorsdottir, 1991). Whereas the Ad5 E1B promoter was activated by Ad40 E1A, Ad5 E1A, and the strong VZV transactivator IEp140 (Everett, 1987), only with the last transactivator could significantly increased expression be obtained from the Ad40 promoter, and even then the level was only 1.4-fold, compared to 8-fold for the Ad5 promoter. It was not possible to discern differences using the truncated plasmids in this assay (not shown).

Recombinant viruses

Our previous studies using recombinant Ad5 viruses expressing the Ad40 E1B region under the control of either the Ad5 or the Ad40 E1B promoter (Bailey *et al.,* 1994) had also shown that the Ad40 promoter was much weaker than its Ad5 counterpart, but interpretation was complicated by variation in posttranscriptional process-

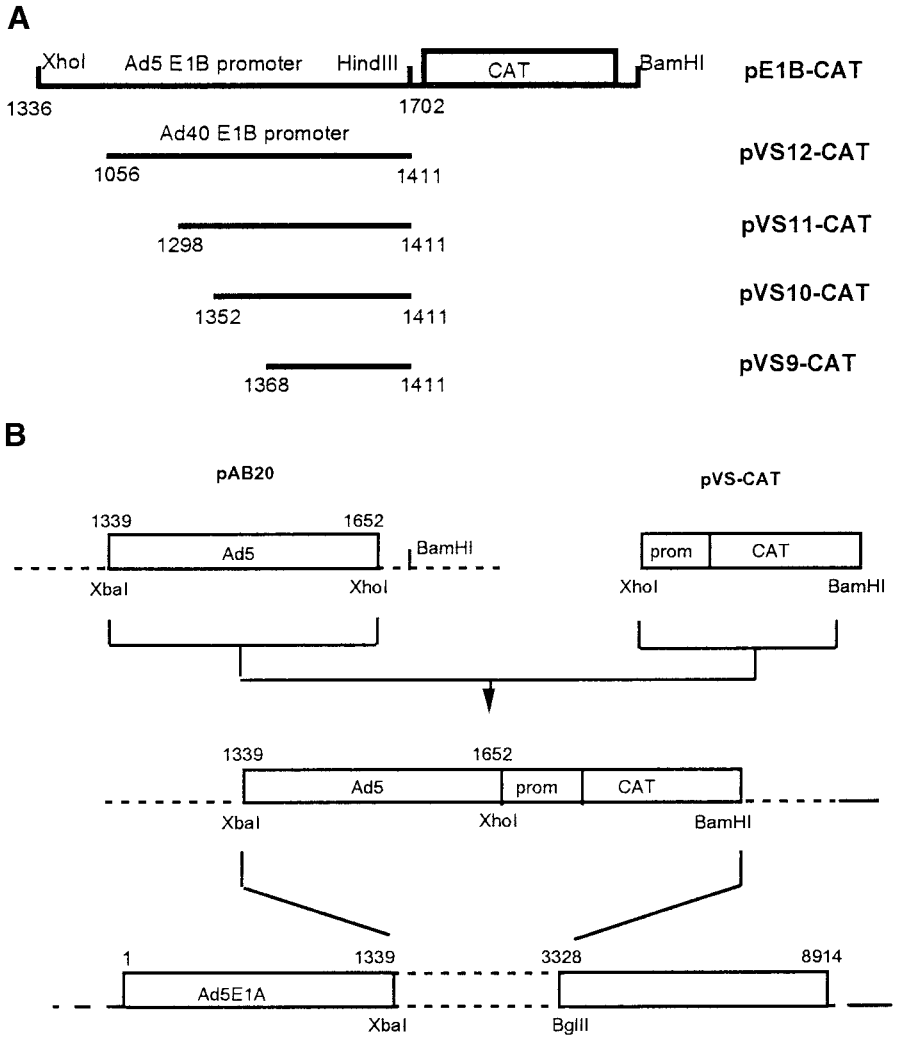
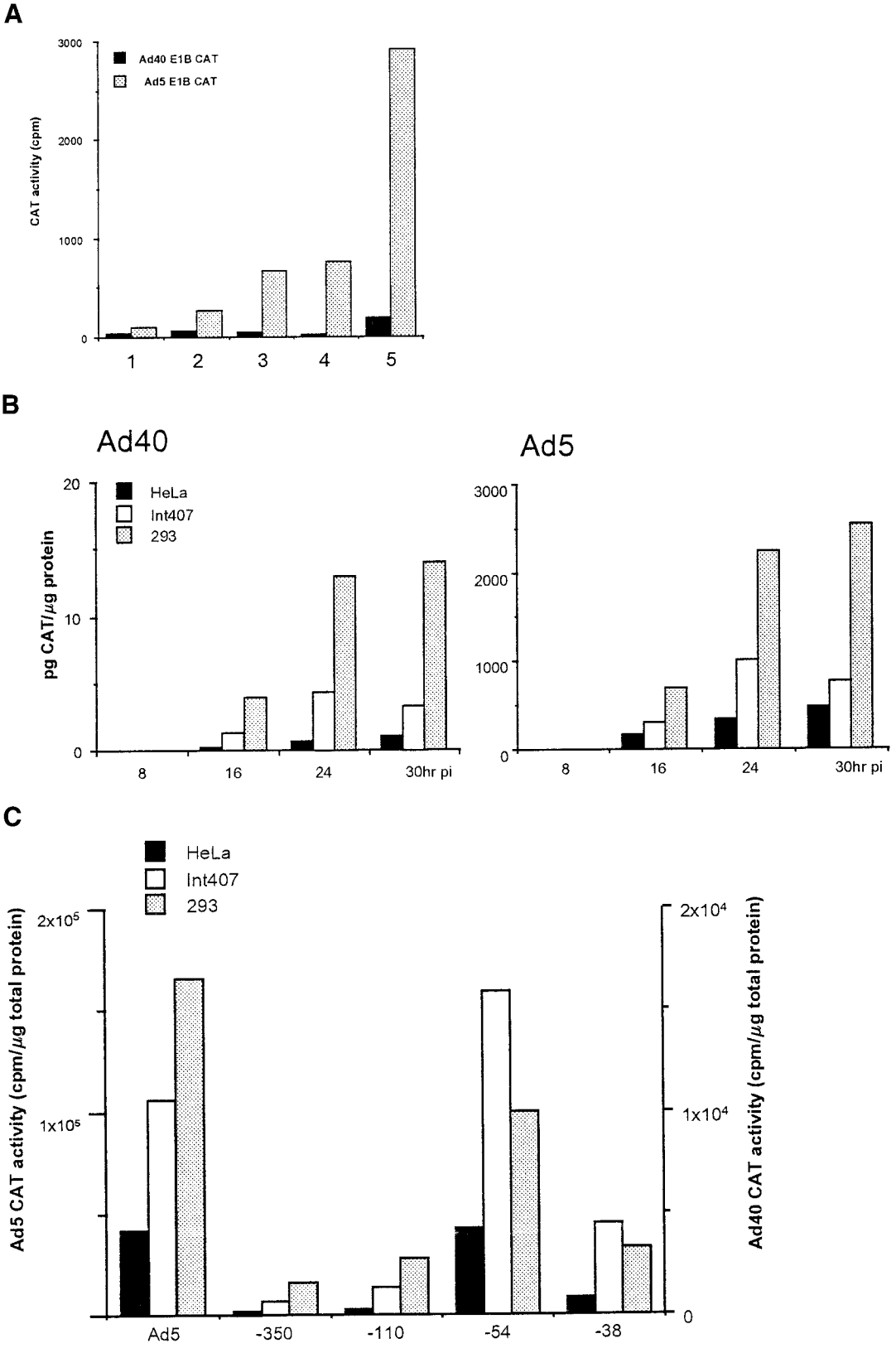


FIG. 2. Ad40 E1B promoter constructs. (A) To generate the pVS-CAT series, the plasmid pE1B-CAT containing the Ad5 E1B promoter was cleaved with *Xho*I and *Hin*dIII, and the Ad5 promoter region was replaced by the full-length Ad40 E1B promoter (pVS12-CAT) or truncations from the 5′ end as shown. (B) To generate plasmids with the Ad5 E1A region upstream of the various E1B promoter/CAT constructs, the promoter-CAT fragment from the pVS series was inserted between the *Xho*I and the *Bam*HI sites of pAB20, and the *Xba*I–*Bam*HI fragment moved into pAB3 (which encodes Ad5 nt 1–1557 and 3328–8914) to give the pAB-CAT series, equivalent to the pVS-CAT series, but with the Ad5 E1 sequences 1–1652 upstream of the Ad40 E1B promoter and CAT coding region.

ing between Ad5 and Ad40 E1B primary transcripts, so quantitation of the difference was not informative. In order to facilitate measurement of transcriptional activity in a virus context, Ad5 recombinant viruses were designed with the Ad40/CAT gene sequences in place of the Ad5 E1B gene, such that E1A mRNA processing signals were unaltered, and effects of altering E1B transcriptional control sequences could be measured in terms of CAT expression. The substitutions made to effect these changes are detailed under Materials and Methods and summarized in Fig. 2B. A control virus, with only the Ad5 E1B coding region replaced by the CAT gene (vE1BCAT), was also made. The viruses were propagated in 293 cells, which complement for the deleted E1B functions, so they grow to the same titer as Ad5 WT. In HeLa cells, although virus yield was not measured, the time course of DNA replication was the same as for Ad5WT (not shown), as would be expected for Ad5 E1B

FIG. 3. (A) Transient transfection assay for E1B promoter activity. Plasmids containing the CAT gene under the control of the Ad5 promoter (nt 1336–1702) or the Ad40 promoter (nt 1057–1411) were transfected into HeLa cells in the absence of a transactivator (1) or in the presence of Ad40 E1A (2), Ad5 E1A (3), Ad5 E1A 13S (4), or VZV IEp140 (5). Cells were harvested at 48 h posttransfection and cell extracts assayed for CAT activity (Seed and Sheen, 1988). (B) Infectivity assay for E1B promoter activity. Ad5 dl309 with the E1B region substituted by the CAT gene under the control of the Ad40 E1B promoter in vVS12-CAT (left) or the Ad5 E1B promoter in vE1B-CAT (right) were used to infect HeLa, Int407, or 293 cells at an m.o.i. of 50



PFU/cell (measured in 293 cells). Cells were harvested at the indicated times, and cell extracts were assayed for CAT activity. (C) Infectivity assay for Ad40 E1B promoter upstream deletions. Ad5 dl309 with the E1B region substituted by the CAT gene under the control of the full-length Ad40 E1B promoter (vVS12CAT; −350) and upstream deletions (vVS11CAT; −110: vVS10CAT; −54: vVA9CAT; −38) were used to infect HeLa, Int407, or 293 cells at an m.o.i. of 50 PFU/cell. Cell extracts taken at 48 h p.i. were tested for CAT activity.

mutants, which can replicate their DNA normally, but are aberrant in the synthesis of late gene products (Babiss and Ginsberg, 1984; Pilder *et al.,* 1986).

The ability of these viruses to express the CAT gene was examined in permissive 293 cells, semipermissive Int407 cells, and nonpermissive HeLa cells. Figure 3B shows a time course of CAT expression using the recombinant viruses vE1BCAT and vVS12CAT (containing full-length versions of the Ad5 and Ad40 E1B promoters, respectively). The most striking feature is that the Ad5 promoter is over 100-fold more active than Ad40, in each cell type. Figure 3B also demonstrates that for both the Ad5 and the Ad40 promoters, a hierarchy of CAT expression is evident among the cell types, with 293 > Int407 > HeLa. The relative values of the CAT yields at 24 h in the three cell types are 1:0.45:0.15 for Ad5 and 1:0.33:0.05 for Ad40, confirming that in addition to the overall lack of responsiveness of the Ad40 sequences, there is a cell type-specific component in the usage of the two promoters, as previously found for recombinants encoding the Ad40 E1B promoter and coding sequences (Bailey *et al.,* 1994).

Promoter deletions

For Ad5, maximal transcription requires the GC and TATA boxes and additional upstream sequences (Spector *et al.,* 1993). To look for possible involvement of upstream sequences in Ad40 E1B promoter activity, the viruses vVS11CAT, vVS10CAT, and vVS9CAT, which bear successive deletions up to the Ad40 TATA box, were tested for CAT expression in all three cell types (Fig. 3C). As before, the complete Ad40 promoter (vVS12CAT) had less than 1% of the activity of the Ad5 promoter, but surprisingly, increased activity relative to the full-length Ad40 promoter was observed with all the constructs bearing upstream deletions. The highest value was recorded when only the GC and TATA boxes were retained (vVS10CAT). Removal of the GC box (vVS9CAT) reduced the activity, but not to the levels seen with the viruses bearing Ad40 upstream sequences (vVS11CAT and vVS12CAT). Moreover, for the two shortest constructs, the hierarchy of cell sensitivity was not maintained, with levels in Int407 exceeding those in 293 cells. The greatest increase was observed in HeLa cells; deleting the 300 bp 5′ to the GC box gave a 37-fold increase in response, compared to 23-fold in Int407 cells and 6-fold in 293 cells. The maximal response observed for the Ad40 promoter was from the GC plus TATA box construct in Int407 cells, where the level attained was 15% of that from the intact Ad5 promoter in the same cells.

Sp1 binding

These experiments suggested that for Ad40, the GC box and TATA boxes are sufficient to obtain maximal response. In contrast to Ad5, however, it appeared that upstream sequences could be playing an inhibitory role. While interpretation of these data might be complicated by considerations of the proximity of the end of the E1A transcript and the alterations in spacing resulting from the deletions, it was nevertheless clear that the Ad40 GC box plus TATA box was some 5- to 10-fold less active than the Ad5 promoter. The TATA box sequences are identical for the two viruses, but the GC box of Ad40 differs in four positions from that of Ad5 and is located closer to the TATA box, with different flanking sequences. In order to show that the Ad40 GC box could in fact bind Sp1, and to assess the efficacy of this interaction, we undertook gel retardation assays using probes derived from the Ad40 and Ad5 sequences and variations thereof. Table 1 lists the oligonucleotides used as double-stranded probes; in addition to Ad40 and Ad5 GC box sequences, there are m4 mutants where all the nonconserved bases have been interchanged, so that Ad40m4 has an Ad5 core with Ad40 flanking sequences, and Ad5m4 has the converse. Ad40m1 and Ad5m1 have reciprocal single base substitutions.

TABLE 1

Oligonucleotide Probes for Gel Shift Assays

Ad40[a]	5′ GTGTTTATTTCTTGGGCGTGTTTGTGGG
Ad40m1	----------G---------
Ad40m4	----------G-----G-GC------
Ad5[b]	5′ CGTGTTAAATGGGGCGGGGCTTAAAGGG
Ad5m1	----------T----------
Ad5m4	----------T-----T-TT--------
Box I[c]	tcgagCCTTACCCTGTGTTTATTTCT
Box II[d]	TCCTTAAAGCGCCCCAAGTGCTCCTGAGATCATAGTAAT

Note. Lowercase letters indicate added restriction site.
[a] Ad40, nt 1345–1372; −58 to −31 relative to Ad40 E1B cap site.
[b] Ad5, nt 1644–1671; −59 to −32 relative to Ad5 E1B cap site.
[c] Ad40, nt 1335–1356; −71 to −50 relative to Ad40 E1B cap site.
[d] Ad40, nt 1285–1323; −123 to −83 relative to Ad40 E1B cap site.

Figure 4A shows gel mobility shifts using the authentic Ad40 and Ad5 probes with nuclear extracts of HeLa and Int407 cells and with semipurified Sp1 protein; in each case there is one major retarded complex, although the nuclear extracts also displayed some minor bands of greater mobility. The fraction of the probe retained in the major complex was calculated after PhosphorImager scanning of the gel and is shown at the bottom of Fig. 4A; comparable levels of DNA binding are seen for the Ad5 probe with the Sp1 sample and the HeLa cell extract, while Int407 extract binds some sixfold less. In contrast, the Ad40 probe bound best to the HeLa extract and least to the Int407 extract.

The specificity of the interaction was tested in competition assays, using a 50- to 200-fold molar excess of unlabeled homologous competitor DNA. The relative efficacy of binding of the variant sequences was measured in the same way, by using the oligonucleotides as unlabeled competitor. Figure 4B shows the binding of the Ad5

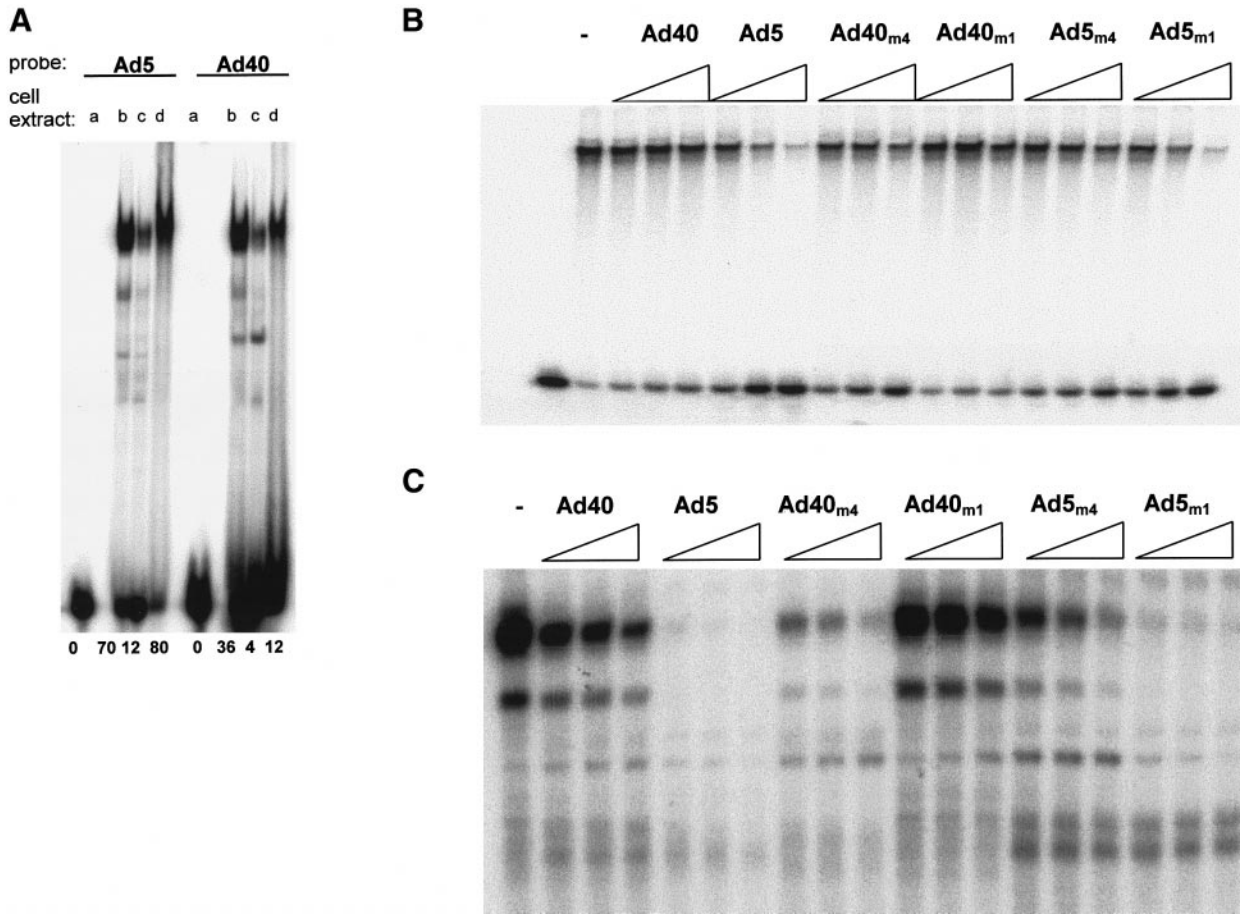


FIG. 4. Gel retardation analysis. (A) Ad5 and Ad40 GC box sequences. 5 μg nuclear extracts of HeLa or Int407 cells or 0.5 μg of a partially purified Sp1 extract was incubated with 1 ng of ^{32}P end-labeled probe (Ad5 or Ad40) at 25°C for 30 min. Free probe and bound probe were separated by electrophoresis on a 3.5% acrylamide nondenaturing gel and visualized by PhosphorImager analysis. The percentage of probe retained in complex as calculated from the digitized image is shown below each lane. (a) No extract; (b) HeLa; (c) Int407; (d) Sp1. (B) Competition for Sp1 binding. 0.5 μg Sp1 extract was preincubated with competitor DNA at 50-, 100-, and 200-fold molar excess for 15 min at 25°C. 1 ng of ^{32}P end-labeled Ad5 probe was added for a further 30 min at 25°C. The competitor sequences are listed in Table 1. (c) Competition for HeLa nuclear extract binding. 5 μg of nuclear extract was preincubated with unlabeled competitor DNA at 50-, 100-, and 200-fold excess for 15 min at 25°C. 1 ng of ^{32}P end-labeled Ad5 probe was added for a further 30 min at 25°C. The competitor sequences are listed in Table 1.

GC box sequence to Sp1: 95% of the input probe was retained in complex, as determined by PhosphorImager analysis. The addition of a 200-fold molar excess of homologous oligonucleotide reduced the amount of labeled probe in complex to 5%, while addition of a 200-fold excess of Ad40 sequence could reduce the binding to only 77%. Using the Ad5 sequence with a single base change (Ad5m1) made little difference, while replacing the Ad5 core with Ad40 bases (Ad5m4) gave a competitor of intermediate efficacy (52% binding). Reciprocally, Ad40m4, with an Ad5 core, was a better competitor (39%), while Ad40m1 was the least active (83.5%). Equivalent results were obtained using HeLa extracts (Fig. 4C) or Int407 extracts (not shown), although there were additional minor bands, not all of which were removed by all the competitors. If Ad40 was used as the labeled probe, Ad5 was better able to remove Ad40 probe from complex than the homologous Ad40 added in excess (not shown).

The observation that inclusion of sequences upstream of the Ad40 GC box reduced transcription suggested that there might be a binding site for a negative regulator of transcription in this region. DNase protection by HeLa cell nuclear extract showed that in addition to binding at the GC box, two regions upstream were protected from nuclease digestion (Fig. 5); using the 3′ end labeled probe VS10-13 (nt 1352–1411) a footprint spanning the GC box was obtained, while VS11-13 (nt 1298–1411), which extends upstream, revealed two further protected domains, mapping at nt 1338–1355 (box I) and nt 1298–1313 (box II). Box I lies just upstream of the GC box, where there is little homology between Ad40 and Ad5, whereas box II maps to the end of the E1A coding region, where one of the Ad5 upstream transcriptional sites is located, and there is some sequence similarity. A gel retardation assay using an oligonucleotide that spans box II (see Table 1) gave rise to two major retarded bands (Fig. 6, lane E). Competition with the homologous

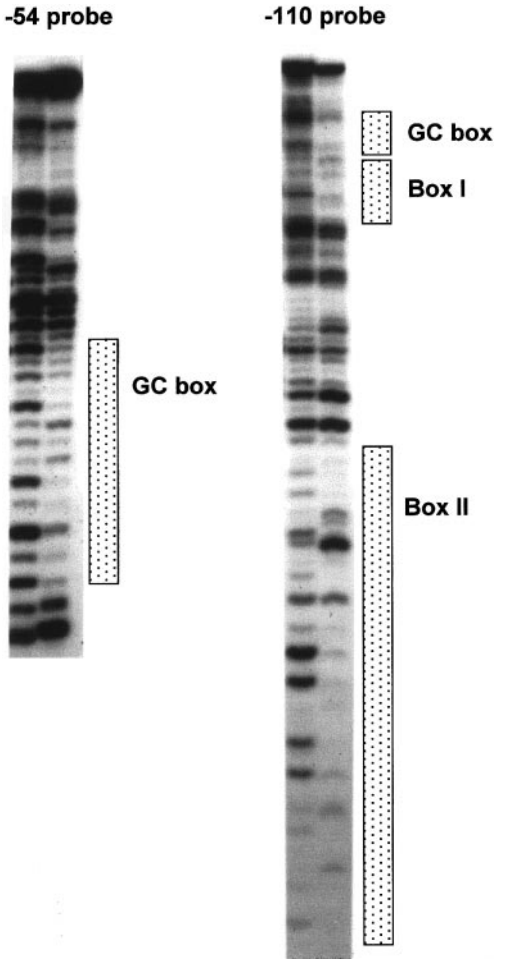


FIG. 5. DNase protection. 5 μl HeLa cell nuclear extract was preincubated with 1 μg poly(dI–dC) · poly(dI–dC) for 10 min at room temperature, followed by a 20-min incubation with 3′ end labeled probe (VS 10-13 and VS11-13), prior to DNase 1 digestion. The samples were analyzed on a 6% denaturing polyacrylamide gel and visualized by PhosphorImager analysis.

Ad40 oligonucleotide competed effectively for the upper band, while an excess of an oligonucleotide covering box I had no effect. The lower band appears to be nonspecific, as neither competitor removed it.

DISCUSSION

The experiments described here were intended to investigate why the Ad40 promoter is apparently so much less active than its Ad5 counterpart, when they have very similar TATA box and GC box sequences. By replacing the Ad40 E1B coding region with the CAT gene, we were able to obtain a quantitative measure of the difference in activity, without the additional complexity imposed by the presence of the E1B coding region, which not only has a complex splicing pattern, but also encodes proteins that play an intimate role in the processing of mRNA. By placing the Ad40 promoter in an Ad5 virus context we could minimize other contributing factors, such as the relatively poor transactivating capacity of the Ad40 E1A 13S protein compared to its Ad5 counterpart (Fig. 3A) and the relative difficulty of obtaining high-titer low-particle-to-infectivity-ratio virus stocks (Steinthorsdottir, 1991; Mautner *et al.,* 1995).

The CAT assays confirmed that the Ad40 E1B promoter is over 150-fold less active than Ad5 in an Ad5 virus background and that the cell type-specific variation in CAT expression paralleled the pattern previously seen in Ad5/40 recombinants, where we measured steady state E1B mRNA levels (Bailey *et al.,* 1994), thus implicating the E1B promoter as the dominant factor affecting permissivity. Use of a partially purified Sp1 protein extract allowed us to confirm that the Ad40 promoter can indeed bind to Sp1 and that Sp1 binding levels in the three cell types tested mirror the CAT activity and hence the promoter activity measured. Gel shifts with a range of heterologous competitors revealed that not only the pre-

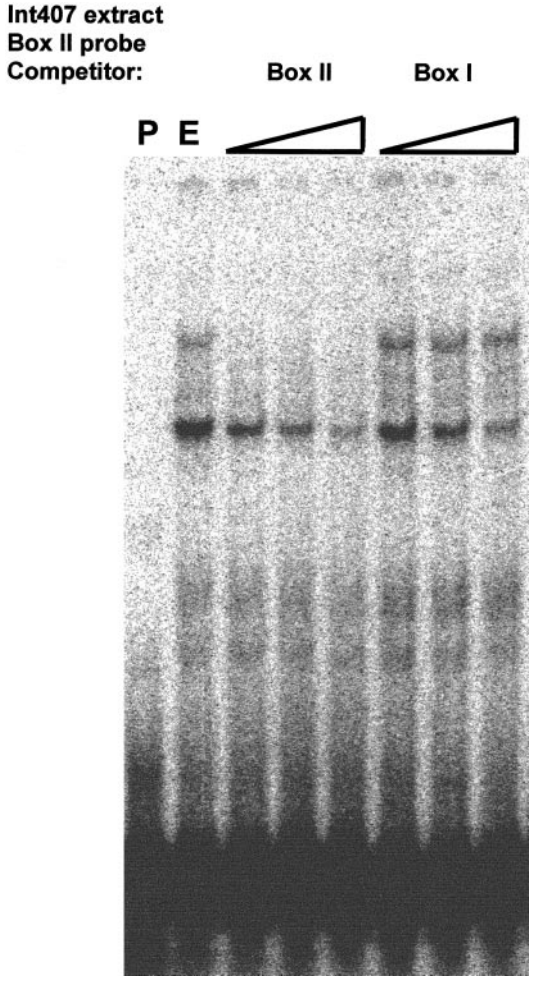


FIG. 6. Gel retardation assay with upstream sequences. 5 μl Int407 cell nuclear extract was incubated with 1 ng of ^{32}P end-labeled probe for box II (nt 1285–1323) for 30 min at 25°C (lane E). Competition by preincubation for 15 min at 25°C with 50-, 100-, and 200-fold excess of homologous box II and with box I (nt 1336–1356) oligonucleotides is shown at the right. The sequences are listed in Table 1.

cise sequence of the GC box, but also the flanking sequences contribute to the strength of binding. From the DNase 1 protection experiment, and the gel shifts with box I and II probes, it is clear that there are other proteins that bind upstream of the GC box. The proximity of box I may also have a negative influence on the binding of Sp1 to the GC box, as its removal enhances transcription significantly.

The surprising finding that the removal of Ad40 upstream sequences allowed more CAT synthesis suggests that whatever binds to these sequences acts as a repressor rather than an activator of transcription. We had tested the Int407 cells as possibly permissive cells for Ad40 on the basis that they were originally described as being derived from fetal gut, and Ad40 has a marked tropism for the gut, particularly of very young infants. Int407 cells are in fact more permissive for Ad40 than HeLa cells, but they have been analyzed by DNA fingerprinting and are almost indistinguishable from HeLa cells on this basis. Whether they express less of the putative E1B repressor protein(s) than HeLa cells remains a possibility, which could help explain the differences we observed. A search of the Transfac transcription factor database (http://transfac.gbf.de/TRANSFAC) offers some interesting candidates for upstream binding proteins for box I. Box I shows very strong similarity to the consensus binding site for transactivators with the winged helix motif, in particular members of the HFH and HNF families, which play a role in controlling tissue-specific gene regulation (Clevidence *et al.,* 1993; Peterson *et al.,* 1997). A testable hypothesis is that in cells that are not the natural host of Ad40, the family member may bind and interfere with transcription, whereas in the appropriate cell type (i.e., intestinal cells) the corresponding family member would possess transactivating properties. Whether box II is a novel silencing component remains to be investigated; there is no strong similarity to any sequences in the Transfac database.

In summary, we can identify five factors contributing to the defective activity of the Ad40 E1B promoter, relative to that of Ad5: (i) a poor response to transactivation, seen not only with the homologous Ad40 E1A but also with Ad5 E1A and with the highly active varicella zoster virus transactivator IEp140 (Fig. 3A); (ii) modulation of the basal activity by the presence of a negative regulatory element in the region −110 to −54 (Fig. 3C); (iii) a GC box that binds Sp1 with a lower affinity than the Ad5 homolog (Figs. 4B and 4C); (iv) sequences flanking the core GC box that may decrease Sp1 binding and activity (Figs. 4B and 4C); (v) the absence of an equivalent of the upstream activation region identified by Spector *et al.* (1993). Further experiments in which Ad5 and Ad40 upstream sequences are interchanged in minimal Ad40 and Ad5 E1B promoter constructs will allow many of these intriguing possibilities to be addressed directly.

TABLE 2

Primers to Generate Ad40 E1B Promoter Truncations

Oligo	Location	Sequence[a]
VS9	1368–1385	5′ ctgcagctcgaGTGGGTATATAAGCAGGT
VS10	1352–1369	5′ ctgcagctcgagTTTCTTGGGCGTGTTTGT
VS11	1298–1315	5′ ctgcagctcgagCCAAGTGCTCCTGAGATC
VS12	1056–1074	5′ ctgcagctcgaGAACAAGAGTTAACGACTT
VS13	1411–1395	5′ ctgcagaagcttGAATAAACTAACACTAA

[a] Uppercase letters indicate Ad40 sequence; lowercase letters indicate added restriction sites.

MATERIALS AND METHODS

Cells and virus propagation

293 cells are human embryonic kidney cells transformed by and expressing the Ad5 E1 region; they were maintained in Eagle's medium containing 10% fetal calf serum. Int407 cells were obtained from the European Collection of Animal Cell Cultures; although originally described as being derived from human embryonic intestine (Henle and Deinhardt, 1957), they are characterized now as HeLa, on the basis of DNA fingerprinting (Gilbert *et al.,* 1990). HeLa and Int407 cells were maintained in Dulbecco's MEM containing 5% fetal calf serum. Recombinant Ad5/CAT viruses were grown and titrated in 293 cells.

Plasmids and viruses

The plasmid pE1B-CAT (Dery *et al.,* 1987) was used as a basis for constructs with the CAT gene under the control of various promoter sequences. pE1B-CAT consists of the Ad5 E1B promoter (nt 1336–1702) inserted between the *Xho*I and the *Hin*dIII sites of pBA-CAT, upstream of a promoterless CAT gene (see Fig. 2A and Dery *et al.,* 1987). Four PCR products encoding full-length or truncated Ad40 E1B promoter sequences were generated using the appropriate primer combinations (see Table 2); these were cloned into pE1B-CAT in place of the Ad5 *Xho*I/*Hin*dIII fragment (pVS-CAT series; Fig. 2A). The DNA sequence of the recombinant plasmids was confirmed by dideoxy sequence analysis.

pAB20 consists of Ad5 nt 1339–1652 (which has an *Xba*I site at the left end), with *Xho*I and *Bam*HI sites added at the right end, cloned into the *Xba*I and *Bam*HI sites of pT7T318U (Pharmacia). The promoter/CAT cassettes from the pVS-CAT series of plasmids and from pE1B-CAT were cloned into pAB20 via the *Xho*I and *Bam*HI sites (Fig. 2B). The *Xba*I/*Bam*HI fragment from these constructs was then moved into pAB3 (Bailey *et al.,* 1994) (which carries Ad5 nt 1–1557 plus nt 3328–8914), replacing the *Xba*I/*Bgl*II fragment, so that the pAB-CAT series has the E1B promoter flanked by the Ad5E1A region and the CAT coding sequences (Fig. 2B). These

constructs were recombined *in vivo* with *Xba*I cut Ad5 dl309, to generate recombinant viruses with the Ad5 E1B promoter replaced by Ad40 sequences and the CAT gene replacing the Ad5 E1B coding sequences (Bailey *et al.,* 1994). Recombinant plaques identified by restriction enzyme analysis were plaque-purified twice more prior to preparation of virus seed stocks.

Transient transfections

Subconfluent HeLa cells in 60-mm plates were transfected using DOTMA (Boehringer Mannheim); for each plate, 4 μg of the CAT reporter plasmid was cotransfected with 4 μg of inducer plasmid or pUC8. Plasmid DNA in 1.5 ml infection medium was mixed with 40 μl DOTMA in 1.5 ml medium, added to the cell monolayer and incubated at 37°C for 6 h. The medium was replaced and incubation continued for a further 48 h. Cells were washed once with PBS, harvested into 3 ml TEN (100 mM NaCl, 50 mM Tris–HCl, pH 7.5, 10 mM EDTA), centrifuged at 2000 rpm for 1 min at 4°C and resuspended in 75 μl of 250 mM Tris–HCl, pH 8.0. Cell were disrupted by sonication and after centrifugation at 12,000 rpm for 2 min the supernatant was used directly for CAT assays (see below).

Virus infections

Cells were infected with recombinant CAT-expressing virus at 50 PFU/cell and harvested at the times indicated in the text. To prepare cytoplasmic extracts, cells (2×10^6/60-mm plate) were washed once in PBS, harvested into 1 ml TEN, pelleted (2000 rpm for 1 min at 4°C), drained, and resuspended in 100 μl of 250 mM Tris–HCl, pH 8.0. After three freeze–thaw cycles, cell extracts were clarified by centrifugation (12,000 rpm for 2 min) and the supernatant was used directly for CAT assays (Seed and Sheen, 1988).

CAT assays

A 20-μl reaction mix contained 10 μl cell extract, 1 μl of 25 mM *n*-butyryl CoA, and 0.5 μl [^{14}C]chloramphenicol (45 mCi/mmol, NEN DuPont) plus 18.5 μl H_2O. After incubation at 37°C for 1 h the reaction mix was extracted with 200 μl of tetramethylpentadecane (TMPD):xylenes (2:1), followed by centrifugation for 1 min in a microfuge. Subsequently, 175 μl of the organic phase was counted by scintillation counting in 4 ml Ecoscint (National Diagnostics). The protein concentration of the cell extracts was measured using the Bradford assay (Bradford, 1976), and CAT activity was expressed as counts per minute per microgram of protein. Where the level of CAT activity has been expressed as picograms of CAT per milligram of protein, then known amounts of chloramphenicol acetyl transferase (Sigma Chemicals Co.) were assayed in parallel with the cell extracts.

Gel retardation assays

Nuclear extracts of HeLa and Int407 cells from confluent monolayers (10^7 cells/140-mm plate, 20 plates) or from HeLaS spinner cultures (10L at 10^4 cells/ml) were obtained using the modified Dignam procedure (Dignam *et al.,* 1983). Cells were lysed by Dounce homogenization in 2 vol of 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, 0.5 mM DTT, the washed nuclei were resuspended in 2 vol buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) and incubated on ice for 20 min, and the nuclear debris was removed by centrifugation at 15,000 *g* for 15 min. Protein concentration was measured with the Bradford assay.

Sp1 protein was synthesized using a vaccinia virus recombinant that overexpresses the human Sp1 gene (Jackson *et al.,* 1990). CV1 cells (5×10^7) were infected at 10 PFU/cell and harvested at 26 h p.i. Washed cells were lysed in 2 ml HS (high-salt) buffer (50 mM Tris, pH 7.5, 0.42 M KCl, 20% glycerol, 10% sucrose, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM PMSF, 1 mM sodium metabisulfite, 2 mM DTT) and centrifuged at 80,000 *g* for 1 h. The supernatant was applied to a 0.4-ml wheat germ agglutinin column (Vector Laboratories) preequilibrated in HS buffer, washed with HS buffer, and then washed with Zn buffer (25 mM HEPES, pH 7.6, 12.5 mM $MgCl_2$, 20% glycerol, 0.1% NP-40, 10 μM $ZnSO_4$, 1 mM DTT, 0.1 M KCl) and eluted with 2 ml Zn buffer containing 0.3 M *N*-acetyl glucosamine. The fractions from the column were adjusted to 50% glycerol and assayed for DNA binding activity in a gel retardation assay and for protein concentration by the Bradford assay.

The standard gel retardation reaction mix of 20 μl contained 1 ng double-stranded (ds) oligonucleotide probe, 5′ labeled with ^{32}P at one or both ends, 1 μg ds poly(dI–dC) · poly(dI–dC), 3 μl buffer C (as above), 5 μl buffer B (25 mM HEPES, pH 7.9, 8 mM $MgCl_2$, 2 mM DTT, 0.04% BSA), and 0.5–15 μg nuclear extract or affinity-purified Sp1. The reaction was incubated at 25°C for 30 min, loaded directly onto a 5% nondenaturing polyacrylamide gel (made with 50 mM Tris base, 306 mM glycine, 0.1% NP-40), and electrophoresed at 260 V for 1.75 h at 4°C. For competition assays, a 50- to 200-fold molar excess of unlabeled double-stranded oligonucleotide was preincubated with the extract for 15 min at 25°C, prior to addition of the labeled probe.

The total protein concentration was chosen to ensure that in standard reactions, over 80% of the probe was incorporated into complex. Dried gels were analyzed on a PhosphorImager, and digitized images were quantitated using ImageQuant software (Molecular Dynamics).

DNase protection assay

HeLa cell nuclear extract (5 μl) was preincubated for 10 min at room temperature with 1 μg poly(dI–dC) ·

poly(dI–dC) in 200 μl DNase 1 buffer (10 mM Tris, pH 7.0, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA, 200 mM KCl, 100 μg/ml BSA, 2 μg/ml calf thymus DNA, 20% glycerol). DNA probes were generated by 3′ end labeling of the *Xho*I site in the DNA fragments VS10-13 and VS11-13. Nuclear extract and probe were incubated for 20 min at room temperature, prior to DNase 1 digestion (concentration and time were determined empirically for each reaction). The reaction was terminated by addition of 7 μl stop buffer [645 μl ethanol, 5 μl tRNA (1 mg/ml), 50 μl saturated ammonium acetate]. The samples was kept on dry ice for 15 min and centrifuged at 20,000 *g* for 10 min, and the pellet was washed with 90% ethanol before resuspension in 5 μl of 95% formamide, denaturation by boiling, and electrophoresis on a 6% denaturing polyacrylamide gel. A G+A ladder, generated by chemical cleavage of the probe fragment, was used as marker.

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