DNA synthesis-dependent relief of repression of transcription from the adenovirus type 2 IVa\textsubscript{2} promoter by a cellular protein

Wenying Huang, J. Kiefer, D. Whalen, and S.J. Flint*

Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA

Received 20 February 2003; returned to author for revision 1 April 2003; accepted 14 May 2003

Abstract

The promoter of the human adenovirus type 2 IVa\textsubscript{2} gene, which becomes active only during the late phase of infection, is built largely from sequences spanning, and downstream of, the sites of initiation of transcription. These sequences comprise an initiator, an intragenic sequence necessary for efficient transcription from the promoter by RNA polymerase II, and an intragenic binding site for a cellular repressor of IVa\textsubscript{2} transcription. The properties of the latter protein, which is termed IVa\textsubscript{2}-RF, suggested that it might account for the viral DNA synthesis-dependent activation of IVa\textsubscript{2} transcription during the adenoviral productive cycle. Here we report the results of experiments to assess the contributions of DNA template concentration and IVa\textsubscript{2}-RF binding to the activity of the IVa\textsubscript{2} promoter using a transient expression system. When an IVa\textsubscript{2}-EGFP reporter gene was introduced into HeLa cells, in which IVa\textsubscript{2}-RF was identified, no EFGP synthesis could be detected. In contrast, in IVa\textsubscript{2}-RF-containing cells in which the plasmid carrying the chimeric gene replicated, synthesis of both the EGFP protein and the IVa\textsubscript{2}-EGFP mRNA was readily detected. A vector mutation that blocked plasmid replication reduced IVa\textsubscript{2} promoter activity to undetectable levels. In contrast, a IVa\textsubscript{2} promoter substitution that impaired binding of IVa\textsubscript{2}-RF increased IVa\textsubscript{2} promoter activity under all conditions examined. Furthermore, introduction of DNA containing the IV-RF binding site with the chimeric reporter genes resulted in increased transcription from the IVa\textsubscript{2} promoter in the absence of plasmid replication. These properties are consistent with the hypothesis that the relative concentration of the IVa\textsubscript{2} promoter and of the cellular repressor that binds to it governs transcription from this adenoviral promoter.

© 2003 Elsevier Inc. All rights reserved.

Introduction

The productive cycle of human subgroup C adenoviruses such as adenovirus type 2 (Ad2) is characterized by strict, sequential expression of viral genes (see Shenk, 2001). This program is established primarily by mechanisms that regulate transcription by RNA polymerase II. The E1A transcription unit includes an enhancer that is recognized by cellular proteins alone (Hearing and Shenk, 1983, 1986). This gene is therefore transcribed efficiently as soon as the viral genome enters the nucleus at the beginning of the infectious cycle. During the early phase of infection, alternative splicing of E1A pre-mRNA produces two predominant mRNAs, which encode 243 and 289 amino acid proteins that differ in the presence of a unique, internal
fected cells (see Flint, 1986; Shenk, 2001). In fact, initiation of viral genome replication once E2 proteins have attained an appropriate intranuclear concentration leads to three distinct types of transcriptional alteration. In the first place, three viral promoters (pIX, IVa2, and E2 late) that are silent during the early phase of infection become active (see Flint, 1986; Shenk, 2001). In addition, transcription of the major late (ML) transcription unit terminates close to the right end of the genome (Fraser et al., 1979), rather than at many sites within a large sequence near the middle of the transcription unit, the pattern observed prior to viral DNA synthesis (Akusjärvi and Persson, 1981; Iwamoto et al., 1986; Shaw and Ziff, 1980). The mechanism by which termination of ML transcription is regulated is not known, but the switch in termination might be governed by differences in the association with proteins of entering viral DNA molecules and those produced within the infected cell. Finally, the efficiency of ML transcription increases some 20- to 30-fold with the transition into the late phase of infection (Shaw and Ziff, 1980).

Such late phase-specific stimulation of ML transcription requires promoter sequences located at positions +86 to +96 (DEF1) and positions +113 to +124 (DEF2) of the ML transcription unit in both in vitro transcription systems and infected cells (Jansen-Durr et al., 1988; Leong et al., 1990; Mansou et al., 1986; Mason et al., 1990). These sequences are recognized by proteins present only in infected cells (Jansen-Durr et al., 1988, 1989; Leong et al., 1990; Mondesert et al., 1992). The protein termed DefB, which binds to DEF2, is a dimer of the viral late IVa2 protein (IVa2p), whereas DefA, which binds specifically to DEF1, contains IVa2p and one or more additional, infected cell-specific proteins that have not yet been identified (Lutz and Kedinger, 1996; Tribouley et al., 1994). The IVa2 protein is the only sequence-specific transcriptional activator encoded within the adenoviral genome, although its sequences required for DNA binding do not conform to those of any well-characterized DNA binding motif (Lutz and Kedinger, 1996). Stimulation of ML transcription by the IVa2 protein in infected cells remains to be demonstrated directly, in part because IVa2p also is responsible for recognition of the viral DNA packaging signal and therefore essential for assembly of virus particles (Zhang and Imperiale, 2000, 2003). However, the observation that the IVa2 protein stimulates ML transcription in a transient expression assay via the internal promoter sequences listed above (Tribouley et al., 1994) is consistent with such a role. Such dependence of efficient ML transcription on synthesis of the IVa2 protein would imply that the crucial step in establishing the late-phase transcriptional program in adenovirus-infected cells is DNA synthesis-dependent activation of transcription from the IVa2 promoter.

In previous studies, we identified a cellular protein that represses IVa2 transcription by binding to an internal sequence of the viral promoter (Chen et al., 1994; Lin and Flint, 2000). The promoter sequence contacted by this repressor, which is currently termed IVa2-RF, lies between, and is partially superimposed on, both the initiator element and an internal sequence essential for efficient initiation of IVa2 transcription (Chen et al., 1994; Lin and Flint, 2000). Adenovirus infection does not result in reduced activity of IVa2-RF (Lin and Flint, 2000). We therefore proposed that the IVa2 promoter becomes active in infected cells only when viral DNA synthesis increases its concentration above that at which all copies can be bound by the repressor. Here we report the results of experiments demonstrating DNA synthesis-dependent titration of the repressor of IVa2 transcription in a simplified experimental system.

**Results and discussion**

**Replication-competent plasmids containing IVa2-EGFP reporter genes**

To test the hypothesis that the activity of the Ad2 IVa2 promoter is controlled by the relative concentrations of the cellular repressor of its transcription, IVa2-RF, and the binding sites for this protein in the IVa2 promoter, the latter promoter was introduced into a plasmid vector that can replicate in mammalian cells under appropriate conditions. This vector contains the SV40 origin of replication, which is recognized by SV40 large T antigen (LT), the only viral protein required for replication from this origin (Difflay, 1992; Fanning, 1994). The Ad2 IVa2 promoter, or a mutant derivative (Rep6) containing a substitution that decreases the affinity of IVa2-RF for its binding site some five-fold (Lin and Flint, 2000), was introduced upstream of an EGFP reporter gene in the SV40-origin containing vector (Fig. 1), as described under Materials and methods.Sibling vectors that cannot replicate even in the presence of LT were constructed by mutation of the SV40 origin binding for LT, which is essential for replication from this origin (see DePamphilis, 1993; Fanning, 1994). This mutation inhibited synthesis of vector DNAs in LT-producing cells as expected (see Fig. 5B). These origin-lacking vectors also provided controls for any effects of SV40 LT on the activity of the adenoviral IVa2 promoters: LT is not only the origin recognition protein of SV40, but also activates viral late transcription (Brady et al., 1984; Keller and Alwine, 1984) and can stimulate transcription from many promoters in transient expression assays (Damania and Alwine, 1996).

**The Ad2 IVa2 promoter is inactive in uninfected HeLa cells**

The activity of the IVa2 promoter was first examined in Hela cells, in which IVa2-RF was identified (Chen et al., 1994). These cells do not contain coding sequences for SV40 LT and therefore cannot support replication of the vectors described in the previous section. The SV40 origin-containing plasmids were introduced into HeLa cells by the
calcium phosphate coprecipitation method and EGFP autofluorescence was examined 24 to 72 h later, as described under Materials and methods. The strong, constitutive, human cytomegalovirus immediate-early promoter in the same vector was introduced into parallel cultures as a positive control. A significant fraction of the cells that received the latter plasmid exhibited a strong EGFP signal (Fig. 2). In contrast, no cells into which the wild-type (wt) IVa2-EGFP reporter gene was introduced synthesized such high concentrations of the protein, and only a small number exhibited much weaker EGFP autofluorescence at any time examined (Fig. 2). Furthermore, the Rep6 promoter mutation did not increase the number of cells synthesizing EGFP (data not shown).

Our in vitro analyses of the organization of the IVa2 promoter employed HeLa cell extracts competent for transcription by RNA polymerase II (Kasai et al., 1992; Chen and Flint, 1992; Chen et al., 1994). Such systems support quite efficient IVa2 transcription, but only at specific ratios of extract proteins to template DNA concentrations: as the concentration of HeLa cell proteins was increased above an optimal value, the efficiency of IVa2 transcription was observed to decrease sharply (Leong and Flint, 1984), a property subsequently attributed to inhibition by IVa2-RF (Chen et al., 1994). The results described here indicate that the HeLa cells contain a sufficient concentration of IVa2-RF to block transcription from even the relatively large number of IVa2 promoters that is likely to be introduced into individual cells by DNA-mediated transformation. They are therefore consistent with one crucial tenet of the repressor titration hypothesis described in the introduction.

COS-1 cells contain IVa2-RF

Cells of the COS-1 line, which was derived from African monkey kidney CV1 cells, synthesize large quantities of SV40 LT constitutively and support DNA synthesis from the SV40 origin of replication (Gluzman, 1981). To determine whether these cells were suitable for analysis of the effects on DNA replication on the activity of the Ad2 IVa2 promoter, we assayed whole-cell extracts prepared from them for IVa2-RF activity, using the electrophoretic mobility shift method described under Materials and methods. When COS-1 cell proteins were incubated with a synthetic, 32P-labeled DNA fragment containing the IVa2-RF binding site of the IVa2 promoter, a complex of low mobility that comigrated with a complex formed by HeLa cell proteins was observed (Fig. 3, lanes 2 and 8). The unlabeled IVa2 DNA sequence effectively blocked formation of both the HeLa and the COS-1 protein–DNA complexes, but a DNA fragment containing the Rep6 mutation, which impairs binding of human IVa2-RF (Lin and Flint, 2000), was a much less effective competitor (Fig. 3, compare lanes 3–6 to lane 2 and lanes 9–12 to lane 8). These data establish that...
simian COS-1 cells contain IVa2-RF activity. Moreover, the results of experiments in which the formation of the IVa2-RF complex was examined as a function of extract protein concentration (data not shown) indicated that the concentrations of the repressor in HeLa and COS-1 cell extracts were similar.

Replication-dependent activity of the IVa2 promoter in COS-1 cells

To investigate the effects of increased intracellular DNA concentration on control of IVa2 promoter activity by IVa2-RF, the SV40 origin-containing and -lacking plasmids carrying chimeric IVa2-EGFP genes were introduced into COS-1 cells, and reporter gene expression was examined initially using EGFP autofluorescence. In contrast to the results obtained with HeLa cells, a substantial fraction of COS-1 cells receiving wt IVa2-EGFP in the origin-containing vector exhibited moderate-to-strong fluorescence (Fig. 4). The exact proportion of such EGFP-synthesizing cells varied from experiment to experiment (Table 1). Nevertheless, efficient synthesis of EGFP required the presence of a functional origin of replication in the vector: the mutation that eliminated the origin both substantially reduced the number of cells in which EGFP could be detected (Table 1) and the strength of the fluorescent signal in those cells synthesizing EGFP (Fig. 4). We can therefore conclude that expression of the chimeric IVa2-EGFP gene in COS-1 cells varied from experiment to experiment (Table 1). Nevertheless, efficient synthesis of EGFP required the presence of a functional origin of replication in the vector: the mutation that eliminated the origin both substantially reduced the number of cells in which EGFP could be detected (Table 1) and the strength of the fluorescent signal in those cells synthesizing EGFP (Fig. 4).

The activity of the IVa2 promoter carrying the Rep6 substitution was also compared to that of the wild-type. This mutation reproducibly resulted in synthesis of EGFP in a slightly higher fraction of cells, but this increase was too small to be considered significant (Table 1). Because the Rep6 mutation decreases the affinity with which IVa2-RF binds to the IVa2 promoter by some five-fold, and increases the efficiency of IVa2 transcription in vitro, this minimal effect of the Rep mutation was somewhat unexpected. However, it seemed possible that the reporter genes reached sufficiently high concentrations in the period before EGFP autofluorescence was examined to obscure any difference in the initial rates of transcription from the wild-type and mutant promoters. Furthermore, although convenient and rapid, examination of EGFP autofluorescence does not provide a quantitative measure of reporter gene expression and hence promoter activity. For example, the quantities of EGFP produced in individual cells varied considerably (e.g., Fig. 4), a parameter that is difficult to quantify, and ignored in a simple count of cells positive above a particular (subjective) threshold. Such considerations prompted direct examination of the synthesis of chimeric IVa2-EGFP mRNAs in cells containing the wild-type and Rep6 reporter genes.

Cytoplasmic and nuclear fractions were prepared from COS-1 cells into which the various vectors had been introduced and RNA was purified from the former fraction as described under Materials and methods. Chimeric IVa2-EGFP mRNA was assayed using primer extension from a primer complementary to a 25-nucleotide sequence within the EGFP coding sequence. Low-molecular-mass DNA was isolated from the nuclear fraction of each sample, and the vector DNA was examined by hybridization to an internal fragment of the EGFP gene following transfer of the DNA to nylon membranes, as described under Materials and methods. The results of one such experiment are shown in
Fig. 5, and data from several such experiments are summarized in Table 2.

The 89-nucleotide-long cDNA product predicted for primer extension of the chimeric mRNA was detected in RNA preparations made from COS-1 cells into which the SV40 origin-containing vectors were introduced (Fig. 5A, lanes 3, 4, 7, and 8). However, no chimeric mRNA was evident in the corresponding RNA preparations from cells that had received the origin-lacking plasmids (Fig. 5A, lanes 5, 6, 9, and 10). As illustrated in Fig. 5B, the vector DNAs were nevertheless present in the nuclei of such cells, although, as expected, at substantially lower concentrations than those attained by the origin-containing DNAs. These results therefore confirmed that the activity of the IVa2 promoter is governed by promoter concentration. Furthermore, the results obtained when the activity of the Rep6 mutant promoter was compared to that of the wild-type established that this property is not merely a result of the greater ability of the viral promoter to compete for components of the cellular transcriptional machinery when its concentration is increased by plasmid replication. The Rep6 mutation resulted in a significant increase in chimeric mRNA concentration compared to that observed in cells containing the wild-type IVa2-EGFP reporter gene (Fig. 5A; compare lanes 3 and 7 and lanes 4 and 8). In this experiment, the vector containing the mutant promoter accumulated to a slightly higher concentration than its sibling carrying the wild-type (Fig. 5B). However, this difference was far too small to account for the ~10-fold difference in the corresponding chimeric mRNA concentrations determined by PhosphorImager analysis (Fig. 5A; Table 2).

An internal control plasmid was introduced into COS-1 cells with the IVa2-EGFP-containing vectors in experiments similar to that shown in Fig. 5. However, we invariably observed synthesis of concentrations of control mRNAs too low for reliable quantification, regardless of whether an RSV-neomycin or an HCMV-luciferase reporter gene was used. Such poor expression of widely used reporter genes was presumably a result of the low concentrations of their promoters, which were introduced into COS-1 cells in non-replicating plasmids, in the cells that also contained high concentrations of the IVa2-EGFP reporter genes. To obtain a quantitative measure of the effects of the Rep6 mutation, we therefore determined the concentrations of both the chimeric IVa2-EGFP mRNA and the vector DNA using the methods described above. In these experiments, only the

---

Table 1

Expression of an EGFP reporter gene from wt and mutant IVa2 promoters in the absence and presence of plasmid replication

<table>
<thead>
<tr>
<th>IVa2 promoter</th>
<th>SV40 ori</th>
<th>% Cells EGFP positive</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>Ori-</td>
<td>2.8</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>wt</td>
<td>Ori+</td>
<td>21.0</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>Rep6</td>
<td>Ori-</td>
<td>4.5</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Rep6</td>
<td>Ori+</td>
<td>23.4</td>
<td>14.2</td>
<td></td>
</tr>
</tbody>
</table>

* Cells containing 15 μg of the vectors indicated exhibiting moderate-to-strong autofluorescence were scored as positive.
SV40 origin containing vectors were employed, and such parameters as quantities of DNA were introduced and time of incubation following DNA introduction were varied. The data collected in such experiments, one of which is shown in Fig. 6, were used to calculate ratios of reporter mRNA concentrations to those of the DNA template. Under all conditions tested, IVa2-EGFP mRNA synthesis normalized in this way to template concentration was more efficient from the Rep6 than from the wild-type IVa2 promoter (Table 3). The mutation increased the activity of the IVa2 promoter from three- to nine-fold under the various experimental conditions (Table 3). As the magnitude of this difference could not be clearly correlated with any particular experimental parameter, the variability observed is likely to be due to differences in the efficiencies with which cells took up DNA and in the rates of plasmid replication in different experiments. Regardless, the greater efficiency of reporter gene expression from the Rep6 promoter observed in every experiment establishes that IVa2-RF represses IVa2 transcription in vivo.

The methods employed in these studies assess the total concentrations of mRNA or DNA accumulated during the period in which cells containing the vector DNAs were incubated. Nevertheless, the patterns of IVa2-EGFP mRNA concentration per unit of DNA as a function of time are consistent with the view that the IVa2 promoter becomes active only above a certain threshold concentration. This parameter increased more or less continuously when expression of the EGFP gene was controlled by the Rep6 promoter (Fig. 6), suggesting that the promoter concentration attained by the time the analysis began was sufficient to support the maximal rate of transcription. In contrast, the chimeric

![Fig. 5. Analysis of IVa2-EGFP mRNA synthesis and vector DNA concentration in COS-1 cells. (A) Cytoplasmic RNA was purified from COS-1 cells 48 h after introduction of quantities of the vectors indicated and IVa2-EGFP mRNA was examined by primer extension. The position of the cDNA synthesized from this mRNA is indicated by the arrow at the right and the lengths of the 32P-labeled DNA markers run in lanes 1 and 11 are listed at the left. Lane 2 shows the products of primer extension of cytoplasmic RNA prepared from COS-1 cells into which no exogenous DNA was introduced. (B) Vector DNA present in the nuclear fractions obtained from the samples receiving 15 μg vector DNA shown in (A) was examined by hybridization to a 32P-labeled, internal fragment of the EGFP coding sequence.](image)

![Fig. 6. Accumulation of IVa2-EGFP mRNA and vector DNA in cells containing the wt and Rep6 IVa2 promoters. COS-1 cells that had received 2.5 μg of the SV40 origin-containing vectors carrying wt (A) or Rep6 (B) IVa2-EGFP genes were harvested after the periods incubated. In this experiment, time zero was 12 h following addition of the DNA-containing precipitate. The chimeric reporter mRNA and vector DNA were assayed as in the experiment shown in Fig. 5. The cDNA (mRNA) and vector DNA (DNA) PhosphorImager signals were expressed relative to those obtained for the wt IVa2-EGFP at 24 h and are shown in relative, arbitrary units.](image)

### Table 2
Relative chimeric mRNA concentrations

<table>
<thead>
<tr>
<th>IVa2 promoter</th>
<th>SV40 origin</th>
<th>Relative (IVa2-EGFP mRNA) a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Expt. 1</td>
</tr>
<tr>
<td>wt</td>
<td>Ori−</td>
<td>ND</td>
</tr>
<tr>
<td>wt</td>
<td>Ori+</td>
<td>1.0</td>
</tr>
<tr>
<td>Rep6</td>
<td>Ori−</td>
<td>16.3</td>
</tr>
<tr>
<td>Rep6</td>
<td>Ori+</td>
<td></td>
</tr>
</tbody>
</table>

ND = not detectable.

a mRNA signals are expressed relative to that obtained with the wt IVa2-EGFP reporter in the SV40 origin-containing vector.

### Table 3
Comparison of the activities of the wt and Rep6 IVa2 promoters

<table>
<thead>
<tr>
<th>Quantify vector DNA introduced, μg</th>
<th>Time of incubation, h</th>
<th>(IVa2-EGFP mRNA)/(vector DNA) a</th>
<th>Rep6:wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>15.0</td>
<td>48</td>
<td>23.6</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>24</td>
<td>11.7</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>48</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>24</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>12</td>
<td>3.7</td>
</tr>
</tbody>
</table>

a Arbitrary units.
mRNA accumulation as a function of concentration of the wild-type IVa2-EGFP DNA template increased substantially only during the latest period examined (Fig. 6). This property indicates that some threshold concentration of the IVa2 promoter, at which all copies cannot be bound by IVa2-RF, must be attained to permit transcription from it. As a further test of the conclusion that the relative concentrations of IVa2-RF and the promoter govern IVa2 transcription, we sought to decrease the effective concentration of the repressor by introduction of its DNA-binding site. The IVa2 promoter was inactive whenever its intracellular concentrations were limited to those introduced by IVa2-RF DNA in trans. The SV40 origin-lacking plasmids containing the wild-type (wt) or Rep6 IVa2-EGFP reporter genes were introduced into COS-1 cells with the molar excess concentrations of IVa2-RF DNA indicated, and EGFP autofluorescence was examined 48 h later. The means and standard deviations of two independent experiments are shown. Higher concentrations of IVa2-RF DNA led to decreased synthesis of EGFP, presumably as a result of cytotoxicity.

Materials and methods

Cells, plasmids, and DNA-mediated transformation

HeLa and COS-1 cells were maintained in monolayer cultures in Dulbecco’s modified Eagle’s medium (Gibco-BRL) supplemented with 10% calf serum and 10% fetal calf serum (Gemini), respectively.

A SacII Ad2 DNA fragment extending from positions +32 of the IVa2 to position +131 of the ML transcription units (Fig. 1) was ligated into the multiple cloning site of SV40 origin-containing plasmid pEGFP-1 (Clontech). Products with the IVa2 promoter upstream of the EGFP coding sequence were identified by restriction endonuclease digestion. A plasmid containing the Rep6 mutant IVa2 promoter (Fig. 1) was constructed in the same manner. Derivatives of these plasmids, which are designated pWTVa2EGFP-Ori+ and pRep6IVa2EGFP-Ori+, respectively, with nonfunctional origins of replication were then isolated: the essential origin binding site for SV40 large T-antigen was replaced by an unrelated sequence by the unique site elimination method (Deng and Nicholson, 1992), and the recovery of the mutation was confirmed by sequencing. Plasmid DNAs were purified from Escherichia coli by the Qiagen maxiprep protocol and their quality was checked by electrophoresis in 1% agarose gels cast and run in TAE.

The vector DNAs were introduced into HeLa or COS-1
cells at ~70% confluence by the calcium phosphate coprecipitation method (Graham and van der Eb, 1973). Various concentrations of vector DNAs, 2.5 μg internal control DNAs and 25 μg salmon sperm DNA, were mixed prior to precipitation. Cells were incubated with the precipitates for 10 min at room temperature, and fresh medium was then added. Following incubation at 37°C, the medium and precipitate were removed and the cells were washed twice with phosphate-buffered saline (Gibco-BRL) prewarmed to 37°C, prior to addition of fresh medium. In most experiments, this step was performed 18 h after addition of the DNA-containing precipitates and was defined as time zero. Incubation at 37°C was continued for the periods indicated in the figure legends and tables.

Electrophoretic mobility shift assay for IVa2-RF

Whole-cell extracts were prepared in parallel from actively growing HeLa and COS-1 cells as described previously (Chen and Flint, 1992; Leong and Flint, 1984). Protein concentrations were determined by the method of Bradford (Bradford, 1976). A synthetic, double-stranded DNA fragment containing the sequence of the IVa2-RF binding site of the IVa2 promoter (positions +2 to +35) was 32P-labeled using [γ-32P]ATP (3000 Ci/mmol, NEN) and polynucleotide kinase. Binding of IVa2-RF to this DNA was examined under the conditions described previously (Chen et al., 1994). Some binding reactions contained varying concentrations of unlabeled competitor DNAs with the wild-type sequence or carrying the Rep6 substitution (Lin et al., 1994). Electrophoresis and autoradiography were as described (Chen et al., 1994).

Analysis of mRNA

COS-1 cells into which vectors carrying chimeric IVa2-EGFP genes had been introduced were harvested after various periods of incubation up to 72 h, where time zero was defined as described in a previous section. Cells were washed with ice-cold phosphate-buffered saline (Gibco-BRL) and cytoplasmic and nuclear fractions were separated by extraction with NP-40 as described (Kasai et al., 1992). Total RNA was purified from the cytoplasmic fraction by digestion with proteinase K and phenol/chloroform extraction. The IVa2-EGFP mRNA was assayed by extension from a primer complementary to positions +64 to +89 of the coding strand of the chimeric transcription unit, as described previously (Kasai et al., 1992). The specific primer extension products were quantified using a Molecular Dynamics PhosphorImager.

Analysis of plasmid DNA concentrations

Low-molecular-mass DNA was purified from the nuclear fractions described in the previous section by the Hirt procedure (Finnen et al., 2001; Hirt, 1967). The DNA samples were linearized by digestion with HindIII, denatured, and loaded onto nylon membranes as described previously (Finnen et al., 2001; Hirt, 1967). Membranes were then hybridized to an internal fragment of the EGFP coding sequence labeled by the random priming method (Feinberg and Vogelstein, 1983). Signals were detected by autoradiography and quantified using a Molecular Dynamics PhosphorImager.

Acknowledgments

We thank Norma Caputo for preparation of the manuscript. This work was supported by a grant from the National Institute of General Medical Science, National Institutes of Health.

References

Fraser, N.W., Nevin, J.R., Ziff, E., Darnell, J.E., 1979. The major late adenovirus type-2 transcription unit: termination is downstream from the last poly(A) site. J. Mol. Biol. 129, 643–656.


