The Downstream Regulatory Sequence of the Adenovirus Type 2 Major Late Promoter Is Functionally Redundant

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Mutagenesis of promoter sequences and oligonucleotide competition assays have been used to demonstrate that late-phase-specific stimulation of the adenovirus type 2 major late promoter is mediated by functionally redundant elements located between positions +75 and +125. These octamer motif-related sequences are recognized by multiple factors.

Among the changes in the transcriptional program that mark entry of the adenovirus productive cycle into the late phase is a dramatic and specific increase in the rate of transcription from the major late (ML) promoter (23, 29). Such late-phase-specific activation of ML transcription was initially shown by Mansour et al. (16) to require downstream elements. It is now clear that adenovirus-infected cells contain one or more factors that recognize at least three downstream promoter elements that increase the efficiency of ML transcription (8, 9, 14). One infected-cell-specific downstream element factor, termed DEF, which recognizes the sequence consisting of nucleotides +86 to +95, has been characterized in some detail, and its binding to the ML promoter has been implicated in stimulation of transcription (9). Nevertheless, our understanding of the mechanism responsible for late-phase-specific stimulation of ML transcription is far from complete. It is not known whether virus-encoded factors, cellular proteins modified as a consequence of adenovirus infection, or cooperating viral and cellular proteins mediate this effect, nor has an exhaustive catalog of relevant factors been made. For example, the relationship of DEF to factors recognizing other downstream sequences which have been identified by DNase I footprinting (9, 14) is not yet clear. In an attempt to address such issues, we undertook a detailed analysis of the downstream elements of the ML promoter and the factors that bind to them.

The effects of mutations of downstream sequences on ML promoter activity were analyzed by *in vitro* transcription with whole-cell extracts. Templates containing the ML promoter were linearized by restriction endonuclease cleavage at downstream sites and transcribed, under conditions empirically determined to be optimal for detection of stimulation of ML transcription, in the presence of [α - 32 P]GTP. Under these conditions, ML transcription was completely inhibited by 1 μ g of α -amanitin per ml (data not shown). As reported previously (8, 9), extracts prepared from HeLa cells harvested 18 but not 4 h after adenovirus type 5 (Ad5) infection transcribed an ML promoter comprising sequences from nucleotides -506 to +192 7- to 20-fold more efficiently than extracts prepared in parallel from uninfected HeLa cells (e.g., Fig. 1B, lanes 2 and 3). Such increased transcriptional activity was not a nonspecific consequence of infection, for

the human *c-myc* P2 promoter showed no increase in activity when transcribed in late-phase infected-cell extracts (data not shown). In these runoff transcription assays, the linear ML templates yielded both the expected runoff transcript and a second product shorter by some 10 bases (Fig. 1). ML transcripts made *in vitro* or in infected cells generated identical primer extension products from position +35 (data not shown). The shorter transcript (Fig. 1) must therefore be the result of specific, premature termination, as reported previously (e.g., see references 12, 15, and 32). As the two products generated in runoff transcription assays represented specifically initiated ML transcripts, both were included in quantitative analyses.

Analysis of the activities of a series of ML templates truncated between positions +70 and +192 in uninfected- and Ad5-infected-cell extracts indicated that deletion of sequences between positions +70 and +140 reduced the ratio of infected-cell extract to uninfected-cell extract activities from 6.9 to 1.5 but had no effect on the absolute efficiency of transcription in uninfected cell extracts (data not shown), in agreement with a previous report (9). In an effort to delineate more precisely the elements required for stimulation of ML transcription, the effects of linker-scanning mutations introduced at roughly 10-bp intervals between positions +10 and +140 on promoter activity in infected-cell extracts were examined. Only mutations located between positions +86 and +126 reduced ML transcription (Fig. 1A), and then by no more than a factor of approximately 2 (as judged by direct counting of transcripts from gels like that shown in Fig. 1A). These modest changes in ML transcription efficiency were reproducible in independent preparations of late-phase infected-cell extracts. As it was possible that the more drastic effects of deletion of sequences downstream of position +70 in comparison with the linker-scanning mutations were some trivial result of juxtaposition of plasmid and ML sequences, we examined the activities of templates carrying internal deletions in mock-infected- and infected-cell extracts. Internal deletion of nucleotides +35 to +65 (the +35/+65 deletion) or of nucleotides +75 to +125 induced little change in the production of ML transcripts by HeLa cell components (Fig. 1B); quantitation of the ML transcripts of these templates and correction for their substantial differences in GMP content indicated that the +35/+65 and +75/+125 deletion templates were 125 and 80%, respectively, as active as the wild type. However, the +75/+125 deletion severely inhibited the stimulation of transcription observed in late-phase infected-cell extracts, reducing the ratio of infected- to uninfected-

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FIG. 1. Effects of downstream linker-scanning mutations and internal deletions on the activity of the ML promoter in Ad5-infected-cell extracts. (A) Templates carrying linker-scanning (LS) (20) mutations at the positions indicated at the top and the wild-type template pML described in the text were linearized by cleavage with *Hind*III at position +192 of the ML promoter and transcribed in whole-cell extracts prepared as described previously (13) from HeLa cells harvested 18 h after Ad5 infection. The transcription reaction mixtures contained, in a volume of 50 μ l, 0.02 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.9), 16 mM $(\text{NH}_4)_2\text{SO}_4$, 5 mM MgCl_2 , 1 mM dithiothreitol, 10% glycerol, 25 μ M each ATP, CTP, and UTP, 12.5 μ M GTP plus $[\gamma\text{-}^{32}\text{P}]\text{GTP}$, 8 μ g of DNA template per ml, and 3.2 mg of extract protein per ml. The transcripts were purified as described elsewhere (10) and analyzed by electrophoresis in 8% polyacrylamide sequencing gels (18). (B) Transcription reaction mixtures, which were as described for panel A, contained whole-cell extract protein from uninfected-cell (lanes 2, 4, and 6) or 18-h-infected-cell (lanes 3, 5, and 7) extracts prepared in parallel and wild-type template (lanes 2 and 3) or templates with internal deletions of nucleotides +35 to +65 (lanes 4 and 5) or +75 to +125 (lanes 6 and 7). All templates were linearized by *Hind*III cleavage at position +192, and runoff transcripts were analyzed as described for panel A. End-labelled *Hpa*II fragments of pBR322 used as size markers were loaded in lane 1. Sizes are given in nucleotides on the left.

cell extract activities by a factor of about 6 (from 21.1 to 3.2 in the experiment whose results are shown in Fig. 1B). These *in vitro* results are in good quantitative, as well as qualitative, agreement with those of a previous *in vivo* analysis (14).

The failure of any one linker-scanning mutation within the ML promoter region from nucleotides +75 to +125, whose deletion severely impaired late-phase-specific stimulation of ML transcription (Fig. 1B), to induce more than a modest inhibition of transcription (Fig. 1A) suggested that this promoter segment might contain several independent elements, each resulting in no more than a twofold stimulation of transcription, or might comprise functionally redundant elements. Consistent with the latter possibility, this segment of the ML transcription unit contains both an imperfect, inverted repeat centered near position +100 and two sequences related to sites recognized by the ubiquitous octamer-binding factor Oct1 (5, 19, 24, 25, 30) (Fig. 2A). Each of the latter sequences exhibits as good a match to the consensus octamer motif as bona fide Oct1-binding sites do to one another (e.g., see references 1, 2, 5, 11, and 30). To test the second hypothesis more directly, the abilities of oligonucleotides comprising positions +75 to +105 or +105 to +135 to inhibit ML transcription were examined. Both oligonucleotides specifically inhibited ML transcription when added to extracts prepared from infected cells harvested during the late phase of infection (Fig. 2B), indicating that one or more positively acting factors recognize sequences included within these oligonucleotides. Moreover,

each oligonucleotide inhibited ML transcription by a factor of 5 to 6 at the highest concentration tested; these reductions were similar in magnitude to that observed when the entire sequence between positions +75 and +125 was deleted (Fig. 1B). This result, together with those of the mutational analyses described in previous paragraphs, strongly argues that the +75/+125 region of the ML promoter required for efficient, late-phase-specific stimulation of transcription contains functionally redundant elements. The octamer-related sequences illustrated in Fig. 2A are the only elements common to the two oligonucleotides and are therefore the most likely candidates for such functionally redundant elements. We therefore sought to identify the factor(s) that might operate via such redundant elements.

To facilitate examination of the several specific factors detected in whole-cell extracts, 0.1, 0.3, and 1.0 M KCl fractions recovered by phosphocellulose chromatography of uninfected- and Ad5-infected-cell extracts were assayed in electrophoretic mobility shift assays. Equal quantities of uninfected- and infected-cell proteins were incubated with double-stranded oligonucleotides, which had been purified, annealed, and end labelled with polynucleotide kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described previously (3), under conditions identical to those used in the transcription assays (except that ribonucleoside triphosphates were omitted). The products were then analyzed by electrophoresis at 4°C in 4% polyacrylamide gels cast and run in 50 mM Tris, 380 mM glycine, and 2 mM EDTA. Comparable concentrations of three specific complexes were observed when the reaction

FIG. 2. Functional redundancy of downstream elements of the ML promoter. (A) Sequence features of the +75/+125 region of the Ad2 ML transcription unit. The inverted and octamer-related repeats discussed in the text are indicated by the arrows and boldface type, respectively. HSV IE, herpes simplex virus immediate-early gene TAATGARAT motif. (B) Inhibition of ML transcription by oligonucleotides containing downstream sequences. Transcription reaction mixtures, as described in the legend to Fig. 1, contained whole-cell extract proteins from infected cells, linearized pML template, and the indicated molar excess concentrations of the +75/+105, +105/+135, or nonspecific (NS) oligonucleotide. The oligonucleotides were incubated with template DNA and extract proteins for 10 min at 30°C before addition of ribonucleoside triphosphates.

mixtures contained the +75/+105 oligonucleotide described in a previous paragraph and uninfected- or infected-cell 0.1 M KCl fraction proteins (data not shown). Unique to the infected-cell 0.1 M KCl fraction was a factor forming a novel, specific complex which displayed a mobility very similar to that of the complex containing DEF described previously (9) and whose binding site is included in the +75/+105 oligonucleotide. None of the four specific complexes formed between this ML segment and 0.1 M KCl fraction proteins was effectively inhibited by the +105/+135 oligonucleotide (data not shown). When the +105/+135 oligonucleotide was used as a probe, one major complex inhibited by the homologous oligonucleotide (Fig. 3A), but not by +75/+105 DNA (data not shown), was formed with 0.1 M KCl phosphocellulose fraction proteins obtained from uninfected and Ad5-infected cells (Fig. 3A, complex d). However, a second complex of slightly greater mobility was also detected when the binding reaction mixtures contained infected-cell proteins. As the total concentration of +105/+135 DNA was increased in such reaction mixtures, a second complex migrating just ahead of complex d increased in concentration and was then inhibited (Fig. 3A). The faster-migrating complex, whose formation could not readily be detected when the reaction mixtures contained uninfected-cell proteins (Fig. 3A, lanes 2 to 6), might represent binding of a novel, infected-cell-specific factor or a modified form of the factor present in complex d.

The +75/+125 region of the ML transcription unit contains octamer-related sequences (Fig. 2A), and the ubiquitous octamer-binding factor is recovered in the 0.1 M KCl phosphocellulose eluate (4). A specific complex, very similar

in electrophoretic mobility to complex d, was observed when 0.1 M KCl fraction proteins were incubated with a labelled oligonucleotide containing a consensus octamer sequence from the simian virus 40 enhancer (2) (data not shown). Moreover, addition of polyclonal antibodies specific for Oct1 (31), but not addition of preimmune serum, to reaction mixtures containing the +105/+135 oligonucleotide and 0.1 M KCl fraction proteins converted complex d to a more slowly migrating form (Fig. 3B). These results indicate that the well-characterized factor Oct1 binds to a site between positions +105 and +135 of the Ad5 ML transcription unit and raise the question of whether Oct1 (or the other specific binding factors detected in this fraction) plays any role in ML transcription. Such a role is not precluded by the small changes in ML promoter activity in uninfected-cell extracts induced by deletion of these downstream sequences (Fig. 1B and data not shown); for example, all of the transcription assays in this study were performed only with HeLa cell extracts, which support efficient transcription via factors that bind only to upstream promoter elements (26). It is therefore possible that downstream elements such as that recognized by Oct1 permit efficient ML transcription in cell types which are normally encountered by the virus and in which factors operating via upstream promoter elements are limiting.

None of the specific DNA-binding factors detected in the 0.1 M KCl phosphocellulose fraction bound efficiently to sequences present in both the +75/+105 and +105/+135 oligonucleotides, nor does the previously described, infected-cell-specific DEF (9). However, a factor exhibiting this specificity was detected in the 1.0 M KCl fraction. Some of

FIG. 3. Binding of uninfected- and infected-cell 0.1 M KCl phosphocellulose fraction proteins to downstream sequences of the ML promoter. (A) The binding reaction mixtures were as described in the text and contained uninfected-cell (lanes 1 to 6) or infected-cell (lanes 7 to 12) 0.1 M KCl fraction proteins, 0.1 ng of 32 P-labelled +105/+135 DNA, and the relative molar concentrations of +105/+135 DNA indicated. The complex marked **d** at the right exhibited the same mobility and specificity (efficient inhibition by +75/+105 DNA and less efficient inhibition by +105/+135 DNA) as a complex detected with labelled +75/+105 DNA (data not shown). (B) The reaction mixtures were the same as those in the experiment whose results are shown in panel A but contained uninfected-cell 0.1 M KCl fraction proteins and the indicated volumes of preimmune rabbit serum (lanes 2 and 3) or polyclonal anti-Oct1 antiserum (lanes 4 and 5).

the properties displayed by this factor after additional purification by hydrophobic interaction chromatography on phenyl-Superose (Pharmacia) are shown in Fig. 4. The factor, termed octamer-binding factor (OBF), partially purified from uninfected or Ad5-infected HeLa cells harvested 18 h after infection, formed two major complexes with an oligonucleotide comprising nucleotides +75 to +125 of the ML transcription unit (Fig. 4A). Both complexes formed with uninfected- and infected-cell proteins were inhibited by the homologous oligonucleotide (data not shown) but not by an unrelated oligonucleotide (Fig. 4B), and the faster-migrating complex I (Fig. 4A) exhibited a mobility very similar to those exhibited by the single, specific complexes formed on either the +75/+105 or +105/+135 oligonucleotide (data not shown). At low protein concentrations, complex I predominated, whereas the more slowly migrating complex II appeared as the protein concentration was increased (Fig. 4A). Both the +75/+105 and the +105/+135 oligonucleotides, used separately, specifically inhibited formation of both complexes (data not shown), as did the octamer motif-containing oligonucleotide described in the previous paragraph (Fig. 4B). These properties indicate that the sequence consisting of nucleotides +75 to +125 of the ML transcription unit contains two binding sites for OBF, which recognizes sequences related to the consensus octamer motif; complex I results from binding to one of these two sites, and occupancy of the second converts complex I to complex II.

No major difference between the binding properties of OBF partially purified from uninfected HeLa cells and those of OBF partially purified from Ad5-infected HeLa cells could be discerned. The patterns of formation of complexes

I and II as a function of protein concentration were very similar for uninfected-cell and infected-cell OBF (Fig. 4A). The monotonic response to increases in factor concentration observed in both cases (Fig. 4A) and the identical bound lifetimes exhibited by double- and single-occupancy complexes containing HeLa cell OBF (data not shown) indicated that its binding to the two sites is not normally cooperative. Nor did Ad5 infection induce a dramatic change to cooperative binding (Fig. 4A) analogous to that mediated by interaction of the E4 open reading frame 6/7 protein with cellular E2F (6, 7, 17, 22). One difference in the binding properties of proteins present in the OBF-containing fraction partially purified from Ad5-infected cells was the appearance, at high protein concentrations, of a third complex that migrated even more slowly than complex II (Fig. 4A). Like complex II, this third complex formed only on the +75/+125 oligonucleotide and was self-inhibitable (data not shown).

No other sequence-specific factor binding to these ML promoter sequences was detected by electrophoretic mobility shift assays of the 1.0 or 0.3 M KCl phosphocellulose fraction from uninfected or infected cells. Thus, OBF was the only candidate for the factor mediating the function of the downstream repetitive elements of the ML promoter described in previous paragraphs. However, the function of these elements is infected cell specific, whereas OBFs prepared from uninfected and Ad5-infected cells exhibited very similar binding properties (Fig. 4A). Several resolutions of this apparent paradox can be imagined. It is, for example, possible that the electrophoretic mobility shift assay simply failed to detect the infected-cell-specific factor, identical in specificity to OBF, that operates via these downstream

FIG. 4. Binding of uninfected-cell and infected-cell OBF to downstream, octamer-related sequences of the ML promoter. (A) The binding reaction mixtures contained 0.1 ng of ^{32}P -labelled +75/+125 oligonucleotide and the indicated quantities of partially purified OBF prepared from uninfected (lanes 1 to 7) or late-phase-infected (lanes 8 to 14) cell extracts. Formation of the complexes marked I and II at the left was inhibited by the +75/+105, +105/+135, and +75/+125 oligonucleotides (data not shown) but not by unrelated DNA sequences (e.g., lane 4 in panel B). (B) The reaction mixtures contained 0.1 ng of ^{32}P -labelled +75/+125 DNA, uninfected-cell OBF, and the indicated relative molar concentrations of a 30-bp oligonucleotide (octamer) containing an octamer consensus sequence from the simian virus 40 enhancer (2) or an unrelated oligonucleotide (NS).

promoter elements. Alternatively, adenovirus infection might modify the ability of OBF to stimulate transcription rather than its DNA-binding activity. Finally, it is possible that in infected cells OBF interacts with one or more additional factors to form a functional complex, but this complex does not withstand electrophoresis well. At present, we cannot exclude any of these alternatives. However, the third currently appears most consistent with our observations. No factor, other than OBF, that binds with approximately equal affinity to sequences present in both the upstream and downstream halves of the +75/+125 region of the ML promoter was detected by electrophoretic mobility shift assays, and in preliminary footprinting assays only the 1 M KCl fraction from Ad5-infected cells exhibited protection of the octamer-related sequences. In addition, the interaction of Oct1 with the herpesvirus virion transactivator VP16 (e.g., see references 5, 24, and 25) provides a strong precedent for the notion that the activity of an octamer-binding protein might be modulated by physical interaction with a viral protein.

Additional experiments will be necessary to distinguish among these alternatives, to elucidate the separate or cooperative functions of DEF and OBF in stimulation of ML transcription, and to establish the identity of OBF. This factor binds both classes of sequence recognized by Oct1 (Fig. 2A and 4). On the other hand, Oct1 and OBF behave quite differently during chromatography on phosphocellulose (14) (Fig. 3 and 4), and OBF is not recognized by anti-Oct1 antibodies under conditions (Fig. 3B) that permit binding of these antibodies to Oct1 present in the 0.1 M KCl

phosphocellulose fraction. Thus, OBF might represent one of the several additional octamer-binding factors that have been recently described (e.g., see references 21, 27, 28, and 33) or another POU-domain protein. Pit1, for example, has been reported to bind well to both the octamer motif and TAATGARAT sites (31).

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