Identical Genomic Footprints of the Adenovirus EIIa Promoter Are Detected before and after EIIa Induction

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Genomic DNase I footprinting was used to compare specific protein binding to the adenovirus type 5 early, EIIa-inducible, EIIa promoter. Identical protection patterns of the promoter region were observed whether EIIa transcription was undetectable or fully induced. These results suggest that EIIa-mediated transcriptional induction does not increase binding of limiting transcription factors to the promoter but rather that transactivation results from the proper interactions between factors already bound to their cognate sequences.

During the lytic cycle of adenovirus infection, early control of transcription involves the EIIa gene products which transactivate the other early viral promoters. Though the EIIa-mediated activation of the early EIIa gene has been studied extensively (for a review, see reference 1), the molecular mechanism of activation remains unclear. Analysis of deletion and linker scanning mutations (2, 3, 6, 9, 11, 14, 19) within this promoter has indicated that the EIIa responsiveness involves the same host DNA-binding factors as those required for uninduced expression. Recent results (12, 13) have suggested that EIIa transactivation increases the binding of a factor that recognizes the EIIa promoter. However, in a comparable study (16), no increases in in vitro binding activities were detected upon induction. We have used a DNase I sensitivity assay (4, 5, 7, 20) to identify sequences directly involved in the binding of transcription factors to the EIIa promoter in vivo and have examined the effects of the EIIa products on this pattern. Our results support the conclusion that the activation mechanism does not modify the affinity or concentration of a limiting DNA-binding transcription factor.

We measured the levels of EIIa and EIIa expression in cells infected with either wild-type adenovirus (adenovirus type 5 [Ad5]) or d312 (an EIIa deletion mutant of Ad5; Fig. 1) at various times postinfection (p.i.). Cytoplasmic RNA was isolated from Ad5- or d312-infected HeLa cells. Levels of EIIa (Fig. 2A) and EIIa (Fig. 2B) transcripts increased progressively regardless of the presence or absence of hydroxyurea (HU) until 8 h p.i. with the wild-type adenovirus. When replication was allowed to proceed in the absence of HU (−HU), EIIa mRNA levels dropped after 8 h even though EIIa mRNA continued to accumulate in the cytoplasm. In the presence of HU (+HU), both mRNAs continued to accumulate throughout the infection.

The absence of EIIa products resulted in a delayed expression of the d312 EIIa gene, with no EIIa mRNA found at early times (Fig. 2A). EIIa transcripts were barely detectable after 6 h but accumulated at later times p.i. Similar results were obtained previously with in vivo pulse-labeling experiments (15), indicating that these kinetics directly reflect promoter activities. Thus, by collecting the cells at different times p.i., a comparison could be made between an active EIIa-induced gene (Ad5, 6 or 20 h p.i., +HU) and a gene that was either silent (d312, 6 h p.i.) or transcribed (d312, 48 h p.i.) in the absence of EIIa.

If the control of EIIa expression was due to the modulation, by the EIIa products, of the amount or affinity of a critical DNA-binding transcription factor, differences in the DNase I digestion patterns should have been observed when the induced and uninduced genes were compared. HeLa cells were infected with 100 PFU of either Ad5 or d312 per cell, and the nuclei were isolated at 6 h p.i. and treated with DNase I (17). After purification, the DNA was digested to completion with HindIII, and 5-μg samples were fractionated on a 1.5% agarose gel, blotted to nitrocellulose, and hybridized with nick-translated probe A (Fig. 1). A region hypersensitive to DNase I which maps to the promoter region of EIIa at approximately position −50 ± 30 base pairs could be seen in both Ad5 and d312 digestion patterns (Fig. 3), and the kinetics of appearance of these DNase I cuts were very similar in each case. Thus, at this gross level of detection, the digestion pattern of the EIIa promoter appeared to be independent of transcriptional activity, and there were approximately equal amounts of DNase I-sensitive molecules regardless of the presence of EIIa. However, we could not rule out the possibility that minor changes occur upon induction.

Therefore, we examined the DNase I digestion pattern of the EIIa promoter at the nucleotide level (Fig. 4 and 5) by using the high-resolution genomic DNA footprinting assay (5). After DNase I treatment of infected cell nuclei, the DNA was purified and digested with EcoRI and 5-μg samples were fractionated on 8% denaturing polyacrylamide gels, electrobotted to a nylon membrane, and hybridized with highly labeled single-stranded probes (5). With cells infected for 6 h at 100 PFU per cell, several differences between the digestion pattern of the naked DNA and that of the nuclear Ad5 DNA were easily detected on the noncoding strand (Fig. 4; compare lanes 3 and 4 with lanes 14 to 16). As depicted on the right of the figure, the regions protected from DNase I digestion and often bounded by hypersensitive sites correspond to the main EIIa promoter elements defined previously by mutational analysis (2, 14, 19). Additional protections, especially around the cap site, were also detected.

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FIG. 1. Map of Ad5 and schematic of DNA probes. The position of the major capsides (+1) of the Ela, Elia, and EIII transcription units (TU) within the Ad5 genome is represented on the top, with the single-stranded end-labeled DNA probes used to assay mRNA synthesis diagrammed below. The HindIII fragment used to assay gross hypersensitivity of Elia is also represented; numbers below and above it refer to positions relative to the Elia and EIII capsides, respectively. Probe A is the 32P nick-translated probe used to assay gross hypersensitivity. The bottom line shows an enlargement of the Elia transcription unit and the location of DNA sequences cloned in M13mp18 and M13mp19 used in probe synthesis for genomic footprinting experiments (probes B and B', respectively).

suggesting specific protein interactions with this region as well. Cells infected with d312 for 6 h at 100 PFU per cell (Fig. 4, lanes 11 to 13) were also subjected to this analysis, and essentially the same digestion pattern as was obtained for Ad5 was found.

To exclude the possibility that minor protections could have been missed owing to too high a viral copy number present in the nuclei, these experiments were repeated at lower multiplicities of infection (10 PFU of Ad5 per cell, lanes 8 to 10, and of d312 per cell, lanes 5 to 7, Fig. 4), which results in approximately 40 viral genomes per nucleus (data not shown). The same protection profile was observed, and no striking differences could be detected between d312- and Ad5-infected cells. The slightly stronger protection apparent on Ad5 (lanes 8 to 10) has not been observed reproducibly and most likely reflects a slight overdigestion of these samples by DNase I. The digestion pattern of the coding strand was also examined, and, again, no differences between Ad5 and d312 were seen at 6 h p.i. (data not shown).

Since footprints were detected on all essential Elia upstream promoter elements whether the gene was transcribed (Ad5) or not (d312), we conclude that the binding of factors to the DNA is not sufficient for promoter activation.

At 20 h p.i., in the absence of HU, Ad5 replicated, yielding a net increase in viral templates of about 200-fold. At this time, the protected regions on both strands of the promoter were no longer observed (Fig. 5A and B, lanes 4), and the overall digestion pattern was very similar to the naked DNA pattern, with the exception of some residual hypersensitivity at positions −50 and −84. This loss of the upstream footprints late in infection is most likely the result of the dilution of the parental templates by the newly replicated viral DNA molecules which lack detectable proteins bound to the Elia promoter. It was not possible, therefore, to monitor the fate

FIG. 2. Induction of Ela and Elia mRNAs. HeLa cells were infected at 100 PFU per cell with either Ad5 (○) or d312 (△) and cultured in the presence (closed symbols) or absence (open symbols) of 10 mM HU. Cytoplasmic RNA was isolated (8) at various times p.i., hybridized to Elia (A) and Ela (B)-specific single-stranded probes (Fig. 1), and analyzed by S1 nuclease mapping (19). The relative amount of specific transcripts was plotted against time p.i.

FIG. 3. Elia gross hypersensitivity of Ad5 or d312. Shown are results of indirect end-labeling analysis (17) of DNase I-hypersensitive regions present on Ad5 or d312 (dl) at 6 h p.i. DNA (5 µg) prepared from cells infected with Ad5 or d312 and grown in the presence of HU was analyzed by Southern blotting. The lengths of DNA markers (lanes M), in base pairs, are indicated on the left; positions relative to the Elia capsid are shown on the right. The numbers beneath horizontal brackets correspond to the number of minutes of DNase I treatment (25 U/ml, 2.000 U/mg of DNA).
products (Fig. 2A). At these times, no detectable alteration of the digestion pattern of the nuclear d312 genome (Fig. 5A and B, lanes 6 and 7) could be correlated to the increased ELIA template activity when compared with either the silent mutant (6 h p.i.; Fig. 4, lanes 11 to 13) or the active wild-type patterns (20 h p.i.; Fig. 5A and B, lanes 5).

Altogether, these results show that uninduced and induced ELIA promoters exhibit very similar, if not identical, DNase I protection patterns. The protections observed most likely correspond to factors previously identified in vitro and in vivo studies (2, 3, 19): factor A (or E2F [12, 18] or CX [10]), factor B (or ELIA-EF [16] or EPF [18]), and factor C. Preliminary dimethylsulfate footprinting experiments on whole cells confirm these observations (unpublished data) and rule out the possibility that weakly bound factors could have been lost during the preparation of nuclei and could have escaped detection by the DNase I assay. Therefore, it appears that the transcriptional rate-limiting step is not the actual binding of factors to the DNA but rather their ability to form an active transcription complex. Thus, the ELIA-mediated stimulation may be achieved either by the association of additional transcription factors that do not bind directly to the DNA or by the modification of DNA-binding factors. This modification would not result in increased binding of the factors to DNA but would rather allow the establishment of appropriate interactions with the remaining components of the transcription machinery.

This hypothesis is supported further by our recent observations that crude extracts prepared from either d312- or Ad5-infected HeLa cells generated identical complexes with the ELIA promoter, as revealed in vitro by both the DNase I protection assay and the electrophoresis bandshift/dimethylsulfate interference assay (10). These observations, together with those of SivaRaman et al. (16), who found no difference in the ELIA gel retardation pattern upon ELIA stimulation, reinforce our present conclusions. However, these results contradict those reported recently by Nevins and collaborators (12, 13), who detected protein binding to the ELIA region between positions −70 and −30 in extracts from Ad5- but not d312-infected cells (12). As suggested by these investigators (18), at least part of the discrepancy may be related to the use of different types of non-specific competitor DNAs [salmon sperm DNA (12, 18), poly(dI·dI)·poly(dC) (16, 18), or poly(dI·dC) (3, 10)]. In vivo exonuclease III protection experiments have indicated a complex on the ELIA promoter with a 5′ boundary at position −85 only in Ad5-infected, not in d312-infected, cell nuclei (13), although our in vivo DNase I analysis detects proteins bound downstream to position −84 in both cases. Since it is uncertain what causes exonuclease III to stop, the possibility exists that exonuclease III is capable of discriminating between nonproductively bound factors and active transcription complexes. If this is true, then only the DNA engaged in active transcription complexes would confer equal resistance to DNase I.

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FIG. 5. EIIa DNase I footprint on coding and noncoding strands at late times p.i. The hybridization pattern of the genomic blot for noncoding (A) and coding strands (B) of the EIIa promoter is shown. Infections were performed at 100 PFU per cell in the absence (-HU) or presence (+HU) of 10 mM HU. Notations are as described in the legend to Fig. 4. Nuclei from infected cells were prepared at the time indicated above each lane (hours p.i.) and digested for 4 min with DNase I. Viral DNA was hybridized with probe B (A) or B' (B). The same blot was used in both experiments (panels A and B). The autoradiogram shown in panel B was obtained by stripping probe B from the blot and rehybridizing the blot with probe B'.

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