Adenovirus DNA replication facilitates binding of the MLTF/USF transcription factor to the viral major late promoter within infected cells

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ABSTRACT

The activity of the adenovirus major late promoter is substantially increased as the infection proceeds from the early to late phase. To gain insight into the regulation of this promoter, we analyzed protein-DNA interactions by *in vivo* DMS and DNaseI footprinting during the course of adenovirus infection. Little or no protein interaction at promoter sequences was detected early (5 hr) after infection but strong interactions at the major late transcription factor (MLTF/USF) binding site and at the TATA box were evident late (12 hr) after infection. Comparison of *in vivo* and *in vitro* footprints revealed that the *in vivo* interaction late after infection results from binding of the cellular transcription factor MLTF/USF. Nuclear extracts prepared from uninfected cells as well as cells harvested at 5 and 12 hr after infection contained similar levels of MLTF/USF footprint activity, therefore the lack of a detectable interaction early after infection is not due to reduced levels of the factor early in the viral growth cycle. Viral DNA replication was required for MLTF/USF binding at the major late promoter. These results indicate that DNA replication participates in the regulation of adenovirus late gene expression by facilitating the binding of a transcription factor to the major late promoter.

INTRODUCTION

DNA replication and regulation of gene expression are often coupled. Certain transcription factors augment DNA replication (1, 2), and DNA replication can play a role in the transcriptional regulation of gene expression (3–7). Transcription and DNA replication are clearly interrelated in the adenovirus replication cycle. Early after infection, the immediate early gene product E1A transactivates a set of promoters including the major late (ML) promoter whose activity is low and yields RNAs corresponding to the L1 mRNA family (8, 9). Following DNA replication, transcription shows a several hundred fold increase in activity and yields the diverse L1 through L5 mRNA families (9).

What is the molecular basis for the marked change in the activity of the major late promoter during the course of adenovirus infection? DNA sequences upstream (10) as well as downstream (11–13) of the start site have been reported to contribute to maximal expression of the major late promoter. Changes in the *trans*-acting factors bound to these sites could mediate the early to late switch. Moreover, it is likely that the adenovirus chromosome undergoes a modification *in cis* as a result of DNA replication that renders it a suitable template for late transcription (3).

To gain further insight into the regulation of the major late promoter during the course of infection, we analyzed protein-DNA interactions upstream of the cap site of ML gene by *in vivo* DMS and DNaseI footprinting (14–16) both early and late after infection. A significant advantage of the *in vivo* footprinting method is that DNA-protein interactions can be visualized in the genuine chromatin environment. A marked change in the occupancy of the MLTF/USF binding site located upstream of the major late start site occurred as a function of time after adenovirus infection. MLTF/USF was bound to the site late but not early after infection. Efficient binding required viral DNA replication.

MATERIALS AND METHODS

Cells and viruses

HeLa cells were propagated in Dulbecco modified Eagle's medium supplemented with 10% calf serum. Cultures were infected with wild-type adenovirus (Ad5wt300, ref. 17) at a multiplicity of 100 pfu/cell and incubated for 5h, or infected at a multiplicity of 10 pfu/cell and maintained for 12h. In hydroxyurea-treated (10mM), wild-type virus-infected cells and in Ad5ts125-infected (18) cells, a multiplicity of 100 pfu/cell was used.

In vivo footprinting

Monolayer cultures of adenovirus-infected HeLa cells were treated at 20°C for 5 min with 0.5% dimethylsulfate (DMS) in medium without serum (14–16). The reaction was terminated by adding ice-cold phosphate-buffered saline to the cultures,

followed by two washes with the same buffer. DNA was extracted, purified (16) and the beta-elimination reaction was performed with 1M piperidine. The DNA was then cleaved with SacII, the resulting fragments were electrophoretically separated on 6% polyacrylamide gels in 7M urea, transferred to nylon membranes, and hybridized to labeled, single-stranded oligonucleotides corresponding to sequences near the major late promoter as shown in Fig. 1. DNaseI genomic footprinting was performed on isolated nuclei. The enzyme reaction was carried out at 25°C for 3 min at 30 μ g/ml DNaseI. The reaction was terminated by SDS/proteinase K, the DNA was extracted and then analyzed as described for the DMS footprint.

In vitro footprinting

A 375 bp HindIII-RsaI fragment containing the major late promoter was labeled at the RsaI site by polynucleotide kinase. Nuclear extracts prepared from uninfected or adenovirus-infected HeLa cells were incubated with the end-labeled DNA fragment (19), followed by treatment with DMS at 25°C for 3 min at 0.2% final concentration. *In vitro* footprinting was also carried out with partially purified MLTF/USF. Nuclear extracts from uninfected HeLa cells were fractionated by chromatography on a SP-5PW cation exchange column (20). Fractions eluted between 250 and 300 mM NaCl were used. Following the DMS reaction, the DNA was purified, treated with piperidine and size fractionated on sequencing gels.

RESULTS

In our experiments, the rate of transcription measured for the major late transcription unit was about 1000 times higher at 12h than at 5h after infection. In parallel with the apparent increase in transcription rate, the number of adenovirus DNA molecules increased by a factor of 10 as a consequence of the onset of viral DNA replication. As a result, if the apparent activity of the major late promoter is normalized for the number of DNA molecules, it increases by a factor of 100 as the infection moves from the early phase (5 hr) to the late phase (12 hr) of the viral replication cycle. The magnitude of the increase in transcriptional activity is difficult to estimate with precision since only a fraction of viral DNA molecules would be expected to be serving as templates for late transcription at 12 hr after infection. Nevertheless, it is clear that the activity of the major late promoter is substantially increased late after infection.

In vivo MLTF/USF binding late but not early after infection at the major late promoter

To explore the molecular basis for the marked increase in activity of the major late promoter during the late phase of the adenovirus replication cycle, we monitored interactions of cellular factors at the promoter within the infected nuclei by *in vivo* footprinting. Since the supply of cellular transcription factors might be limiting, it was important to keep the number of adenovirus DNA molecules per infected nucleus relatively low and approximately equal when assaying for interactions with cellular factors at early as compared to late times after infection. Accordingly, HeLa cell cultures were infected with adenovirus at a multiplicity of 100 pfu/cell and incubated for 5–6 hr, or infected at a multiplicity of 10 pfu per cell and maintained until 12h after infection. As a result, cells harvested at 5–6 hr after infection contained approximately the same number of nuclear viral DNA molecules as cells harvested at 12 hr; the 10-fold increased input multiplicity of the early sample compensated for the 10-fold increase due to DNA replication in the late sample. Analyses of the levels of intranuclear viral DNA provided direct confirmation that early and late samples contained similar amounts of nuclear viral DNA (data not shown, as an example see Fig. 5, lanes 1 and 2 for comparison).

At 5 or 12 hr after infection, cells were treated with DMS followed by hot piperidine cleavage, or isolated nuclei were prepared and incubated with DNaseI. After either treatment, DNA was purified and cleaved with SacII; DNA fragments were separated by electrophoresis on sequencing gels, electroblotted onto nylon membranes and hybridized to [³²P] labeled, single-stranded DNA probes (Fig. 1). The result of such an analysis is shown in Fig. 2. By using DMS as a footprinting agent, no alteration in G reactivity on the coding strand was observed early after infection (5 hr), as compared to naked DNA treated *in vitro* (Fig. 2, lanes 1 and 2). However, cleavage at G residues on the coding strand was altered late after infection (12 hr) within the binding site for the cellular MLTF/USF transcription factor; some residues were protected and others exhibited enhanced cleavage (Fig. 2, lane 4). DMS-induced cleavage of the noncoding strand was not altered at either 5 or 12 hr after infection (data not shown).

Early after infection (5 hr), DNaseI footprinting showed only a slightly hyperreactive A residue on the noncoding strand (Fig. 2, lane 5) and a protected A residue on the coding strand (Fig. 2, lane 9). In contrast, late (12 hr) after infection DNaseI

Figure 1. Map of the major late promoter of adenovirus. Nucleotide numbers refer to downstream (+) and upstream (–) positions, relative to the major late promoter cap site (nucleotide +1) which is designated by an arrow. Binding sites for MLTF/USF and the TATA binding protein (TBP) are shown. The cap site of the late IVa2 gene is also indicated. Bars at the bottom of the figure indicate DNA fragments or oligonucleotides that were used as probes, and the dotted lines mark the regions analyzed by *in vivo* footprinting.

detected strong interactions on both strands around the binding sites for MLTF/USF and TFIID (Fig. 2, lanes 6 and 10).

Figure 3 summarizes the *in vivo* genomic footprinting results within the portion of the major late promoter at which alterations were detected. It is evident that a major change in protein-DNA interactions occurs with the early to late switch of transcription. Little or no DNA-protein interaction was detected early (5 hr) after infection but strong interactions were detected by both DMS and DNaseI at the MLTF/USF site and were revealed by DNaseI at the TATA box late (12 hr) after infection.

A control experiment was carried out to exclude the possibility that the time dependent changes in DNA-protein interactions were due to the different multiplicities of infection employed to generate early versus late samples. In contrast to the previous experiments, cells were infected with 100 pfu/ml adenovirus and DNA-protein interactions were studied by *in vivo* footprinting late (12 hr) after infection. In these cells the copy number of replicated adenovirus DNA is similar to that of the MLTF which is estimated to be a few thousand per cell (21). Therefore full protection can not be expected, however hyperreactive sites should be visible. The result was in accord with this expectation showing only weak

protection but characteristic hyperreactivity on the coding strand especially at the G residue at the position -61 (Fig. 3) at the MLTF/USF binding site (data not shown).

At the late stage of infection, an extensive footprinting pattern was observed at and around the MLTF/USF binding site *in vivo*. It was important to ascertain whether this pattern was due to interaction of MLTF/USF at its recognition site. The complex formed between MLTF/USF and its binding site has been extensively characterized *in vitro* by DMS and DNaseI footprinting using isolated DNA (21-24). *In vivo* DMS treatment generates a characteristic enhanced cleavage followed immediately by a protected residue within the MLTF/USF recognition site when the factor is bound to the DNA template (Fig. 2, compare lanes 3 and 4). The *in vitro* DMS footprint performed with partially purified MLTF/USF prepared from uninfected HeLa cells (Fig. 4, lane 1) exhibited the same cleavage pattern. This result indicates that the *in vivo* reactivity was, indeed, due to MLTF/USF binding.

Since *in vivo* DMS footprinting demonstrated a difference in MLTF/USF binding during the course of adenovirus infection, we compared the MLTF/USF footprinting activity in nuclear

Figure 2. Autoradiogram of DMS and DNaseI genomic footprinting gels. Protection against and enhancement of the reactions are indicated by symbols. Sequences corresponding to factor binding sites are indicated by boxes. The numbers on the top designate individual lanes on the gels. Footprinting reactions were carried out early (lanes 2, 5 and 9) or late after infection (lanes 3, 6 and 10). DNA from similarly infected cells was isolated and treated with DMS or DNaseI *in vitro* (lanes 1, 3, 7 and 11). The designation G below lanes represents the nucleotide ladder that was used to localize sequence positions in the DNaseI footprints. These samples were isolated from DMS-treated cells late after infection, similarly to the sample loaded on lane 4.

Figure 3. Summary of the *in vivo* DMS and DNaseI footprinting data early as compared to late after infection. The nucleotide sequence corresponds to part of the major late promoter, spanning the MLTF/USF binding site and the TATA box, which binds the TATA binding protein (TBP). Sequences corresponding to these transcription binding sites are framed. Symbols indicating protected or hyper-reactive residues are the same as in Fig. 2.

extracts (10 μ g protein/reaction) prepared from uninfected cells as well as cells harvested at 5 and 12 hr after infection (Fig. 4, lanes 3 to 5). Footprinting patterns were identical by using more extract (13 and 17 μ g protein/reaction)(data not shown). Using less extract (6 μ g protein/reaction) resulted weaker protein-DNA interactions at similar extent with the different extracts (data not shown). We concluded that the different extracts contained similar levels of MLTF/USF footprint activity, therefore the lack of a detectable interaction of MLTF at the major late promoter early after infection is not due to reduced levels of the factor at this point in the viral growth cycle.

Viral DNA replication is required for MLTF/USF binding at the major late promoter in infected nuclei

The timing of the binding suggested that DNA replication is necessary for the interaction of MLTF/USF with the viral chromosome within infected nuclei. To test this possibility, DNA replication was blocked by the addition of 10mM hydroxyurea, a well characterized inhibitor of DNA synthesis (25), and HeLa cells were infected 1hr later with adenovirus at a multiplicity of 100 pfu/ml. As an untreated control, HeLa cultures were infected with 100 and 10 pfu/ml adenovirus for early and late infected cells, respectively. The DMS cleavage patterns displayed in Fig. 5 showed that late (12 hr) but not early (6hr) after infection the MLTF binding site was occupied in untreated cells as observed previously (Fig. 5, lanes 1 and 2, see also Fig. 2, lane 4). MLTF/USF binding was not detected in the presence of hydroxyurea (Fig. 5, lane 3). A similar result was obtained by using the DNA replication-defective Ad5ts125 virus (18) at the nonpermissive temperature (Fig. 5, lane 5). In a control experiment at the permissive temperature with the mutant virus replicating its DNA, MLTF/USF was bound to the major late promoter of Ad5ts125 late after infection (data not shown).

We concluded that DNA replication is required for interaction of MLTF/USF at the adenovirus major late promoter within infected nuclei.

Figure 4. Autoradiogram of *in vitro* DMS footprinting gels. An end-labeled DNA fragment containing the MLTF/USF binding site was incubated either with 4 μ l partially purified MLTF/USF (lane 1) or without added protein (lane 2). Crude extracts (10 μ g) isolated from uninfected, early or late infected cells were also incubated with the labeled DNA fragment, followed by treatment with DMS (lanes 3 to 5). Symbols are the same as in Fig. 2. The asterisk marks a hyper-reactive residue upstream of the TATA box that is evident in the presence of crude extracts.

Figure 5. Autoradiogram of *in vivo* DMS footprinting gels. Cells were infected with 100 (lanes 2, 3 and 4) or 10 (lane 1) pfu/ml of wild type or 100 pfu/ml of mutant (lanes 5 and 6) adenovirus and treated with DMS at 6 and 12 hr after infection as indicated below the lanes. Wild-type (WT) virus-infected cells were treated with hydroxyurea (HU) (lanes 3 and 4) or left untreated (lanes 1 and 2).

DISCUSSION

Our results indicate that, within infected cells, MLTF/USF and TATA binding factor are present at the major late promoter at 12hr but not at 5hr after infection (Fig. 2). Our experiment would fail to detect occupancy of the MLTF/USF binding sites on a small portion of the viral chromosomes present within infected nuclei. In fact, the modest alterations observed for early samples (Fig. 2, lanes 5 and 9) are likely indicative of MLTF/USF binding to a small subpopulation of viral DNAs. Replication of the viral template is required for efficient binding of the factors to the viral chromosome (Fig. 5). Since these factors are required for efficient transcriptional initiation at the major late promoter (21–24), their relative absence early after infection (Fig. 2) is consistent with the low activity of the promoter during this phase of the viral growth cycle.

Our present results are in contrast to the report of Albrecht *et al.* (26), who also used genomic DNaseI footprinting to visualize DNA-protein interactions at the major late promoter. Their assays indicated that the MLTF/USF binding site was occupied early after infection with *dl312*, a mutant virus lacking the E1A gene that is replication deficient (17). We can not as yet explain the apparent discrepancy between the two sets of data.

Viral variants carrying portions of the major late promoter in place of the E1A promoter have been employed previously to identify domains within the late promoter required for its early as compared to late activity (10). A segment including the sequences from –55 to +33 relative to the major late start site with an intact TATA box but missing MLTF/USF site was sufficient for early expression, but failed to activate further late after infection. A second variant with the sequence –122 to +33 containing both the TATA box and MLTF/USF site directed both early and enhanced levels of late transcription. These results are consistent with the idea that MLTF/USF binding is important for late but not early function of the major late promoter — an interpretation that is in complete accord with the *in vivo* footprinting results (Fig. 2 and 5). However, MLTF/USF binding alone is probably not sufficient for the full late response of the promoter since sequences downstream of the start site, which have not been evaluated in our genomic footprinting experiments, also play a role (11–13).

Since there are numerous changes in the adenovirus infected cell as the infection proceeds from early to late phase, we can not unambiguously conclude that the replication process itself is involved directly in the binding of MLTF/USF to its cognate site. However, given the requirement for DNA replication, it seems most likely that binding of MLTF/USF to the viral promoter is somehow linked to the process of replication.

Can DNA replication influence transcription in cellular chromatin by regulating the binding of factors as appears to be the case for MLTF/USF binding on adenovirus chromatin? It has been reported that the rabbit and the *Xenopus* β -globin genes and the *Xenopus* 5S gene display replication dependent transcription (6, 27–31). Although the specific transcription factors involved have not been identified, it has been proposed that the replication process allowed *trans*-acting factors access to the transcription templates. Conceivably, MLTF/USF binding to DNA in cellular chromatin is always linked to ongoing DNA replication. Perhaps some cellular genes whose expression involves MLTF/USF (e.g., 32–34) require DNA replication as a prerequisite for MLTF/USF binding and transcriptional activation.

It is not clear why adenovirus templates that had not been replicated were, for the most part, not associated with MLTF/USF *in vivo*, despite the fact that naked viral DNA can readily bind MLTF/USF. There are several possible explanations. During the early stage of infection the majority of adenovirus DNA molecules might still be within capsids and not accessible to MLTF/USF. At least a portion of the infecting viral chromosomes must be accessible to transcription factors since five early transcription units with promoters spread across the viral genome are actively transcribed by 5hr after infection. Further, all viral templates were readily accessible to DNaseI and DMS by 5 hr after infection (Fig. 2), while encapsidated DNA in purified virions was not accessible to DNaseI and DMS (data not shown). Therefore, it seems likely that the majority of adenovirus molecules are decapsidated and accessible to transcription factors by 5hr after infection. We can also rule out a need to induce MLTF/USF so it can be available to bind to viral DNA, since extracts prepared from uninfected cells or cells infected for 5hr contained the same amount of the factor as cells infected for 12 hr (Fig. 4). Conceivably, MLTF/USF and adenovirus chromosomes are in different nuclear compartments during the early phase of infection, and, as a result, no interaction can occur.

Another factor that could discriminate MLTF/USF binding is the structure of viral chromatin. Non-replicated, input viral DNA molecules do not appear to be associated with the same set of proteins as replicated viral DNA molecules (35,36). The structure of adenovirus chromatin during the early phase of infection might prevent binding of MLTF/USF. This possibility is intriguing since it would predict that some cellular transcription factors, i.e., those responsible for early gene expression can access the early viral chromatin, while others, such as MLTF/USF, can not.

We favor the notion that MLTF/USF does not bind to preformed viral chromatin, but gains access to the viral template during the process of replication as nucleosomes are being disassembled and reassembled. This idea is consistent with earlier work showing that MLTF/USF can not activate a promoter that has been preassembled in chromatin while it can compete for template binding during the assembly process and subsequently activate transcription (37).

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