The appearance of DNase I hypersensitive sites at the 5' end of the late SV40 genes is correlated with the transcriptional switch

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Received 29 July 1981

ABSTRACT

DNase I digestion of the SV40 nuclear chromosome late in infection reveals three hypersensitive sites on the late side of the Bgl I site. Two of these sites at bp 370, 270 correspond to the 5' side of the late transcripts while the third at bp 190, to a region that is required for early transcription. Early in infection, as well as in an SV40 transformed cell line and a T-Ag negative revertant (deleted in the coding region for T-Ag) only one of these sites is present - the one associated with early transcription. Thus, the positions of these major hypersensitive sites are related to the differential expression of the early and late genes. The presence of the characteristic hypersensitive site corresponding to "early" region expression in the revertant, where large T antigen is not synthesized, but where the early "promotor" is intact, indicates that large T antigen is not responsible for this particular hypersensitive site. Additional minor specific DNase I cuts were found on the early genes, at early times only, at 300, 550, 850 bp from Bgl I site. In the transformed cell line, one of these minor cuts is found about 350-400 bp from the Bgl I site and in the revertant, where this region is deleted, a new site is created at 100 bp.

INTRODUCTION

The SV40 genome is associated with cellular histones in a chromatin like structure^{1,2,3} very similar to cellular chromatin^{4,5} and uses cellular enzymatic machinery for its replication and transcription. For these reasons, we chose to use it as a model system to study some aspect of chromatin structure and expression.

DNase I,⁶ endogenous nucleases,^{6,7} restriction endonucleases^{8,9} and micrococcal nuclease¹⁰ all cut, in vitro, the SV40 minichromosome in a region of 400 bp, specifically exposed, located on the late side of the Bgl I site which is located at the origin for DNA replication. This region, which codes only for the agnogene peptide,³⁴ whose function is not known yet, contains also the origin of replication, the T-antigen binding sites^{11,12} and the 5' end of late and early mRNA.^{13,14,15} Repetitive sequences and palindromes¹⁶ whose functions are not yet known are also present in this region.

Similar regions of susceptibility are also observed in cellular chromatin.¹⁷⁻²⁰ These so-called DNase I hypersensitive sites represent localized regions of the chromosome where DNase I introduces a rather precise double-stranded cleavage. As a result, subsequent purification of the DNA restriction endonuclease digestion and blot hybridization using a specific probe results in the appearance of a specific DNA subfragment. The intensity and width of these sub-fragments varies with the probe used. The positions of the cleavages often, but not always map to the 5' side of transcription units, and are correlated with the expression of the gene.²¹⁻²⁴

To establish if the specific sensitivity described in SV40 genome is related to the expression of the early or late genes or both, we used exogenous DNase I to determine the sensitivity of viral chromatin in isolated nuclei at times, when both late and early genes are expressed (late in infection), when essentially early genes are expressed (early in infection, and in the transformed cell, as well as in a revertant).

In the experiments described below, we report that, in the SV40 nuclear minichromosome DNase I hypersensitive sites are located at the 5' end of the early and late genes. Three are present late in infection when early and late genes are expressed. However, only one of them is present at early times, before viral DNA replication, as well as in a transformed cell line and in a flat revertant of that line deleted in T-Ag coding sequences. We correlate these specific DNase I sensitive regions to the differential expression of early and late genes.

MATERIALS AND METHODS

Cell and virus

CV1 monkey cells (provided by J. Champoux) were grown in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum in 75 cm² plastic Petri dishes. Plaque purified wild type SV40 was used to infect cell cultures at a multiplicity of 50 pfu per cell for the experiments done late in infection and at 200 pfu per cell for the experiments done early in infection. 14B and 3-5 cells provided by W. Topp were grown in the same conditions.

Isolation of nuclei and DNase I digestion

Nuclei were isolated according to the method of Groudine and Weintraub,²⁵ or Worcel *et al.*²⁶ The nuclei were resuspended in RSB buffer (0.01 M Tris-HCl (pH 7.4) 0.01 M NaCl 3 mM MgCl₂ 0.5 % Np 40) at a DNA concentration of 0.4 mg/ml and digested 10 min. at 37°C with increasing concentrations of DNase I (10⁻³ µg/ml to 10 µg/ml). The reaction was stopped by adding EDTA to

20 mM and SDS to 0.2 %. The nuclei suspension was then digested 30 min. at 37°C with 20 µg/ml of RNase and overnight with 20 µg/ml of protease K. The phenol-chloroform extracted DNA was precipitated with ethanol and resuspended 10 mM Tris-HCl (pH 7.5) 0.5 mM EDTA and restricted by different restriction enzymes.

Blotting and restriction enzyme digestion

Purified DNA was digested with restriction enzymes (Biolabs) according to the manufactures recommendations and the DNA fragments were separated on 1 % agarose gels. DNA was transferred to nitrocellulose filters (Scheicher and Schuell BA85) according to the method of Southern²⁷ and hybridized to nick translated SV40 DNA probes (S.A. $2-4 \times 10^8$ cpm/µg).

RESULTS

DNase I digestion of SV40 nuclear chromosomes late in infection

In all of the studies to be presented here, the DNase I sensitivity of the SV40 minichromosome was monitored in isolated nuclei. We decided not to use the purified minichromosomes since it is unclear whether purification may lead to subtle rearrangements or to the differential extractions of the various nuclear forms of the minichromosome. Nuclei from SV40 infected cells were isolated late in infection between 16 hr and 18 hr (before the formation of virions, which are resistant to DNase I), and digested for 10 min. at 37°C with increasing concentrations of DNase I. The DNA was then purified and in order to localize specific DNase cuts, it was digested with a variety of restriction enzymes, electrophoresis and Southern transfer hybridization with ³²P SV40 DNA were used to localize specific DNase cuts. Figure 1a shows the results from a typical experiment in which the DNase digested DNA was restricted with Bam H1. Bam H1 makes a single cut in SV40 DNA (at bp 2533) almost mid way between the 3' end of the late and early genes (see map in Fig. 1a). Figure 1a shows the linear 5243 bp SV40 DNA cut only with Bam H1 ; however, as a result of the DNase I digestion, specific SV40 subbands appear. One subband migrates faster and the other slower than the Bam H1-Bgl I digested SV40 marker fragments (Fig. 1a). There is an indication in Fig. 1a that the subbands on either side of the Bam H1-Bgl I markers are doublets. Bgl I makes a single cut in SV40 DNA at the origin of replication (Fig. 1a). A Bam H1-Bgl I digest of SV40 yields two fragments, one of 2710 bp contains the early genes and the other 2533 bp includes the late genes. The fact that the subbands are on each side of the two Bam H1-Bgl I marker fragments, means that DNase I makes specific cuts in the SV40 chromatin near the Bgl I site ; however,

Figure 1. : DNase I digestion of SV40 nuclear chromosomes late in infection.

1a.) Nuclei from infected cells were extracted 16 hr after infection, digested with increasing concentrations of DNase I for 10 min. at 37°C and the purified DNA restricted with Bam HI and separated on 1 % agarose gels, transferred to nitrocellulose paper, and blot-hybridized to ³²P nick-translated whole (left) or early SV40 DNA (right). The central lane shows SV40 markers, Bam HI restricted DNA (5243 bp) together with Bam HI-Bgl I restricted DNA (2710 and 2533 bp). The arrows on the top of the figure indicate increasing DNase I concentrations. The arrows on the map below show where the DNase I cuts occur. Bam corresponds to Bam HI, Bgl to Bgl I.

1b.) The same as in Fig. 1a, except that after DNase digestion, the purified DNA was restricted with Eco RI and separated on 1.3 % agarose gels. The small arrows show the subbands generated by DNase I.

since the DNase I generated fragments do not co-migrate with the markers, it follows that DNase I is not cutting right at the Bgl I site. In order to locate precisely to which side of the Bgl I site these specific cuts are made, we hybridized the same blot to the Bam HI-Bgl I fragment containing the early genes. Only the top subbands are labeled (Fig. 1a, right panel). Similarly, only the bottom subbands are labeled with a late region probe (not shown). These results mean that late in infection DNase I cuts SV40 chromatin specifically on the late side of the Bgl I site. Using the Bam HI-Bgl I SV40 fragments, lambda Hind III fragments, and oX174 Hae III digestion fragments we were able to map the DNase cuts at bp 220 and 390 for this specific experiment (numbers refer to the Buchman, Burnett and Berg numbering system for SV40 DNA sequences : see ref. 16). But in fact, with higher resolute gel and restriction enzyme which yield smaller fragments, as Eco RI (Eco RI makes a single cut on SV40 DNA, see map Fig. 1a), we can resolve three specific DNase cuts, located at bp 190-270 and 370 (see Fig. 1b). The mapping of these cuts were also confirmed using a Taq I digestion (data not shown). This result is in agreement with Wigmore *et al.*²⁸ studies who found several DNase specific cuts on the late side of Bgl I site, late in infection. Control experiment shows that SV40 DNA is not preferentially cleaved by DNase I, at any one of the specific cuts (data not shown).

Figure 1a also shows that the subbands generated by DNase have about the same intensity throughout the DNase I digestion, suggesting that in chromatin specifically cut by DNase I, DNase I digests the early and late genes at about the same rate, although we suspect that only a small proportion of these genomes are active or even potentially active and therefore interpretation of these results is probably impossible.

DNase I digestion of SV40 nuclear chromatin early in infection

To investigate the SV40 chromatin structure before viral replication, when only the early genes are expressed, we digested nuclei from SV40 cells at 7 hr post-infection. In these experiments viral replication did not occur as assayed by the absence of tritiated thymidine labeled viral DNA in the Hirt supernatant²⁹ and by subsequent sucrose gradient analysis (not shown). Early in infection, DNase I introduces specific cuts into SV40 chromatin (Fig. 2). Hybridization with the late Bgl I-Bam HI fragment shows that at early times in infection, DNase I also produces a specific cut on the late side of the Bgl I site (Fig. 2, right panel). In addition DNase also produces specific cuts at 4 hr and 6 hr after infection (results not shown).

There are several features of the digestion early in infection that

Figure 2. : DNase I digestion of SV40 nuclear chromosomes early in infection. Nuclei from infected cells were extracted 7 hr after infection, digested with increasing concentrations of DNase I for 10 min. at 37°C and the purified DNA restricted with Bam H1, separated on 1 % agarose gel, transferred to nitrocellulose paper and hybridized to ³²P nick-translated whole (left) or late (right) SV40 DNA probe. The arrows on the top of the figure indicate increasing DNase concentrations. The arrows on the map show where the DNase I cuts occur.

suggest that DNase I cuts chromatin differently than it does late in infection. Early in infection, a primary cut occurs at about bp 200. When the concentration of DNase I increases, the DNase cutting extends to Bgl I site and still after, the DNase "nibbles" to about 300 bp on the late side and to about bp 5190 on the early side (ie., to about 50 bp on the early side of Bgl I). Using an early Bam H1-Bgl I fragment as probe, three rather regularly spaced specific minor cuts could be detected inside the early genes at 300 bp (number 4900), 550 bp (number 4700) and 850 bp (number 4300) from the Bgl I

site (Fig. 3e). The mapping of all these specific cuts has been confirmed by and R1 restriction analysis of the DNase I digested samples (results not shown).

The subbands generated by DNase I (Fig. 2) are approximately of equal molar intensity at the lower concentrations of DNase I whereas at the higher concentration of DNase I, the intensity of the bottom band increases. This increase is due to the minor cuts inside the early gene, so that at these DNase concentrations the early fragment comigrates with the late fragment genes. Thus, early in infection, like in the late phase, the early genes seem to be digested by DNase I at about the same rate as the late genes, in the chromatin specifically cut by DNase I ; however, again it is not presently possible to assess the significance of this since the percentage of active molecules is not known.

DNase digestion of integrated SV40 in isolated nuclei from SV40 transformed cells

We next asked whether the integrated SV40 genome in SV40 transformed

Figure 3. : DNase I digestion of SV40 nuclear chromosome early (E) and late (L) in infection, hybridized with an early probe.

DNA was restricted with Bam H1 and hybridized with ^{32}P nick-translated early SV40 DNA (the early Bam H1-Bgl I fragment).

cells also contains a DNase I hypersensitive region and whether this hypersensitive region has the fine structure typical of the nuclear SV40 minichromosome present early or late in lytic infection. This is of particular relevance since it is believed that viral expression in transformed cells mimicks that which occurs early in infection (see ref. 16).

For these experiments we used the 14B cell line. These are rat cells transformed by SV40. The choice of this cell line was based on the fact that it contains a single copy of the SV40 genome ; the integration pattern has been well characterized³⁶ (Fig. 4) ; and the integrated genome cloned into λ ³⁶. Integration takes place in the late gene at bp 850. Thus the early genes (Bgl I-Bam H1 fragment) are surrounded by two late viral sequences, at the 5' end, by a 850 bp fragment and at the 3' end, by a DNA fragment of 3779 bp,

Figure 4. : DNase I digestion of isolated nuclei from SV40 transformed cells.

Nuclei of 14B cells were digested with increasing concentration of DNase I indicated by the arrows, on the top of the figure. The purified DNA was restricted with the enzymes indicated on the figure, separated on a 1 % agarose gel, transferred to nitrocellulose paper and hybridized to ³²P nick-translated SV40 DNA. SV40 DNA markers pointed out by arrows (at 5.2 or 2.7 and 2.5 kbp) were added in each digestion. The map shows the integration pattern of the viral genome as described by Botchan et al. (1980). The arrows on the map show the cutting sites for the different restriction enzymes and the small dark arrows, the sites of DNase I cleavage. SST corresponds to SST 1.

which contains an inversion³⁶ (Fig. 4).

Nuclei from 14B cells were isolated and digested with increasing concentrations of DNase I. The purified DNA was then digested with SST (which does not cut the viral sequences) and blot hybridized to SV40. Results from such an experiment are shown in Fig. 4. One can observe that DNase I generates subbands at 4.45 and 3.8 kb from the parental fragment (8.8 kb) of the integrated SV40 genome. Using a variety of restriction enzymes and early SV40 fragment as probe (results not shown), it is possible to map the location of the predominant DNase I cut to bp 230 on the late side of Bgl I (see map Fig. 4). At higher concentration of DNase I, the DNase cutting extends to the Bgl I site. In the SST I digestion, it is possible to see a second subband at 3.8 kb, below the major subband at 4.45 bp (Fig. 4). Using an early DNA fragment as probe, it is possible to show that this second subband is derived from the first one, by a second cleavage in the early gene at about bp 4880. A DNase I cut in the SV40 genome near Bgl I at bp 230, should also generate a fragment which contains the small late region. This fragment is not well resolved on this gel because its size is very close to the size of the other subband. These results are confirmed using Bam HI digested DNA (Fig. 4) where subbands of 4.85 and 2.95 kb are generated from the parental doublet of 7.8 kb. The subband of 2.95 kb which contains the early genes and which migrates in our all experiments slower than the SV40-Bam-Bgl I marker (2.7 kb) locates the DNase cut unambiguously on the late side of the Bgl I site at about 250 bp. This position has been confirmed using Bgl I digested DNA which yields a subfragment of 230 bp (results not shown). A minor cut at Bgl I site generating a fragment of 2.7 kb may also occur at higher DNase I concentrations. One can also observe in the Bam HI digestion, that later in the digestion another subband appears at 2.3 kb as a result of a cut at 400 bp from Bgl I site, inside the early genes. Our data are incompatible with a mapping of this last DNase cut near the Bam HI site. Three bands pointed out by arrows are also visible in the autoradiogram at 5.2, 2.7 and 2.5 kb. These represent marker fragments of SV40 DNA that were added to the DNA samples to insure that transfer and detection efficiency were satisfactory and the migration of DNA in the different lanes were equivalent.

Just above the second band of the Bam HI-Bgl I digestion (4.6 kpb) one can observe a slight subband which is derived from the top band (7.8 kb). The size of this subband indicates that this specific cut made by DNase I is in the adjacent cellular DNA. This site was mapped at 4.8 kb from the viral Bam HI site, on the 3' side of the viral genome. This result is confirmed by

Bgl I digestion (Fig. 4), which produces two viral fragments, one of 4.6 kb, containing the small late fragment and a 24 kb fragment including the rest of the viral genome. A viral subband at 8.2 kb is generated by a DNase I cut in the cellular sequences contained in the 24 kb fragment. This confirms that the DNase I cleaves the flanking cellular DNA, in this SV40 transformed cell line.

DNase digestion of isolated nuclei from revertants

Steinberg *et al.*³⁵ have isolated and characterized T-antigen-negative "flat" revertants from the 14B cell line. We analyzed the revertant (designated 3-5) which has a deletion of 0.8 kb (from bp 4977 to bp 4168, James Stringer personal communication) in the early region and another small one (about 0.2 kb) in the big late fragment, but the early promoter region (see below) is totally intact and should still be functional.

Nuclei from this cell line were digested with increasing concentrations of DNase I and the purified cellular DNA restricted with SST I, Bam HI or Bgl I. It can be observed in Fig. 5 that DNase I generates subbands in the 3-5 revertants. Using Bam HI and Bam HI-Bgl I digestions, we mapped the specific DNase I cuts in the viral genome. The subband containing the early fragment (2.1 kb), in the Bam HI digestion migrates slower than the early fragment obtained after a Bam HI-Bgl I digestion of the control DNA (1.9 kb). This maps the DNase cut on the late side of the Bgl I site at 255 bp. With increasing DNase I, this cut extends to the Bgl I site. Another subband (1.8 kb) appears after, with higher DNase I concentrations, by a cut inside the early Bam HI-Bgl I fragment. Our data are only compatible with a mapping of this cut near the Bgl I site at bp 5150 (ie., -100 bp). The Bgl I digestion shows that there is also a DNase I cut in the flanking cellular sequence in these cells. Adjusting for the SV40 deleted sequences, it appears that two of the three DNase I cuts in this revertant are located at the same sites as those that occur in the parental cell line. However, the cut inside the early gene is closer to the Bgl I site. This cut occurs at about 100 bp in the revertants and at about 350-400 bp from the Bgl I site in the parental cells.

DISCUSSION

The experiments presented here show that DNase I hypersensitive regions on the SV40 minichromosome exist not only *in vitro*^{6,7} but also in isolated nuclei in agreement with previous results³⁷ mainly on the late side of Bgl I site, which is the origin of replication. In addition, we have demonstrated that the location of these specific regions fluctuates during the different

Figure 5. : DNase I digestion of isolated nuclei from revertant 14B cells (5-3). Nuclei from the revertant cells were treated as described in Fig. 5. SV40 DNA markers pointed out by arrow (at 5.2 or 2.7 and 2.5 kbp) were added in each digestion. The cross-hatched region in the map below indicates the deleted sequences.

states of the viral cycle. Late in infection, after the onset of viral DNA replication, when both the early and late genes are expressed, the hypersensitive sites are located exclusively on the late side of Bgl I site, at bp 190, 270 and 370. While this region codes for the agnogene peptide³⁴, the heterogeneous 5' ends of the late mRNA, and the late RNA leader map also in this region.¹³

Early in infection before the onset of viral replication, when the early genes only are expressed and in a transformed cell line and in the T antigen negative revertant where for the most part only early expression occurs³⁸⁻⁴⁰ the location of the hypersensitive sites is slightly different from that observed late in infection. Early in infection, the hypersensitive region includes only one of the hypersensitive sites found in late times, around bp 200-230 and it is more toward the early fragment, includes the origin of

the replication, the different T-antigen binding site,^{11,12} the putative early promoters⁴¹⁻⁴³ and the early 5' end mRNA¹⁴ (Fig. 6). This region also contains the two 72 bp repeats (bp 109-247). It was shown recently by Benoist and Chambon⁴² and Gruss, Dhar and Khoury⁴³, that deletions in one of the two 72 bp repeat inhibit the expression of the early genes *in vivo*. It is interesting to note that this region which is indispensable to the early expression coincides to the region where we found the first DNase I cut (around bp 200-230) on SV40 chromatin early in infection, in the transformed cells as well as in the revertant. Besides the 72 bp repeat, additional SV40 regions between bp 6 and 75 have been shown also essential for early promoter activity.^{41,42,44} The late mentioned regions essential for early expression map in the second part of the early DNase I hypersensitive region, ie. from bp 200 to 0/5243.

These results indicate that there are three major hypersensitive sites ;

Figure 6. : Summary of the different DNase cuts.

The second map from the top shows the different genetic elements included in the hypersensitive regions : (1) The three T-antigen binding sites between bp 61 and bp 5184 (Tjian, 1978) designated by small boxes ; (2) The presumptive early TATA Box bp 14 to 20 ; (3) the 5' ends of the major species of late 19S RNA (bp 292, 373, 526) and late 16S RNA (bp 325) ; (4) the two 72 bp repeats bp 109 to 247 indicated by an empty box.

one when the early genes are expressed, which occurs at bp 200-230 and after extends up to the Bgl I site, and the others which occur after (or during) the onset of viral replication when only the late genes are expressed, are observed at bp 270 and 370. We would like to suggest that this switch in hypersensitive sites reflects the differential expression of early and late genes during the viral cycle, similar to the correlative change in hypersensitive sites and gene activity in several other systems.²¹⁻²⁴

Expression of the early genes is also correlated with minor specific DNase I cuts, in early times at about 300, 550, 850 bp from Bgl site. The first cut maps in the intron of large T antigen and in the coding sequence of a small t antigen (see ref. 16), the second one is in the intron of both large T and small t-antigens, the third is located in the coding region of large T-antigen. These specific cuts do not appear late in infection. One of these cuts is found in the early genes in the transformed cells at about 370 bp from Bgl I site and in the revertant at 100 bp.

Preliminary results show the presence of the hypersensitive region on replicative minichromosome. This fact suggests that the hypersensitivity might be generated and propagated during the replication and not induced by the transcription process, since it has been shown that replicating molecules are not templates for transcription.³⁰⁻³³ Thomas and Mathews⁴⁵ have recently shown that for adenovirus and SV40, late expression depends upon a replication induction in the DNA or chromatin template. We propose that during the viral replication, the promoter region of the late genes undergoes a modification to a more open configuration and that this permits the initiation of transcription. This structural modification is probably not responsible for the binding of the RNA polymerase, because previous studies^{46,47} have suggested that the RNA polymerase is already bound to the late genes in early phase, but that it cannot elongate in vivo. So this change in chromatin structure might facilitate the binding (and/or the accessibility) of other(s) regulatory protein(s) or factor(s) necessary for the initiation of the transcription.⁴⁸

It is interesting to note that in the revertant in which the DNA sequence which contain the minor hypersensitive site are deleted, a new hypersensitive site appears at 100 bp from Bgl I site. These sequences were not hypersensitive to DNase I in the parental cells. The generation of this new hypersensitive site in the revertant might mean that its presence is in some way, necessary in the expression of the early genes. This new hypersensitive site is not at the same distance from Bgl I site as it was in the parental cells (Bgl I site is just taken as a reference point). However, this fact is diffi-

cult to interpret with the data obtained. More specifically, it is difficult to attribute without ambiguity the part which falls to the DNA sequences and the one to the chromatin structure. The deletion might affect the superstructure of the chromatin, which in turn might also affect the location of the hypersensitive site.

The structural basis for the DNase I hypersensitive sites is not known. While it is possible that large T-antigen is responsible for their generations in SV40, we think that this is now very unlikely since the 3-5 revertants retain the hypersensitive site associated with the early "promoter" region even though these cells contain a large deletion in Taq coding region and are T-Ag negative. However, these results do not rule out a cis-acting role for T-Ag in generating late hypersensitive region.

As we mentioned above the DNA sequences might play a role in the generation of the hypersensitive regions, although other factors are clearly involved. For example, Wigmore *et al.*²⁸ using viral mutants duplicated for the hypersensitive site have shown that each of the duplicated region could be hypersensitive to endogenous nuclease. This study shows clearly that this region contains by itself the genetic information to generate a chromatin structure hypersensitive to nuclease. It is interesting to note that when, what we call the late hypersensitive sites are present on the duplicated fragment, the cleavage frequency by DNase I is strongly increased.²⁸

Recently, Ju and Skalka⁴⁹ have pointed out that the Long Terminal Repeat (LTR) of many retroviruses contain region of homology with many other genetic elements particularly 16 bp homologous to bp 124-140 and 194-210 in SV40 genome. These homologous regions are localized in the two 72 bp repeats ; one of which is essential for early expression *in vivo*^{42,43} which is also the region we find the early DNase I hypersensitive site. In addition the LTR region of the active EV-3 endogeneous retrovirus locus in chick cells has also been shown to contain a DNase I hypersensitive site.⁵⁰ These data lead us to suggest that DNA sequences seem to be very important in generating a hypersensitive region by may be providing binding sites for specific proteins and subsequently rendering the binding domain more sensitive to DNase I.

ACKNOWLEDGEMENTS

The author thanks W. Topp for kindly providing the 14B and 3-5 cells, J. Champoux for the CV1, J. Stringer for communication of the mapping of the deletion in the 3-5 cells and A. Larsen for the preparation of the nick translated probes. I thank H. Weintraub for support, encouragement and useful dis-

cussions, and C. Laird, M. Groudine, P. Thomas, J. Lewis and D. Riley for their critical reading of the manuscript. H. Devitt and C. Rtor are acknowledged for typing the manuscript. This work was supported by a grant from N.I.H. to H. Weintraub and by Public Health Service International Research Fellowship number 3 F05 TW02750-0151 to C.C.

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