Volume 13 Number 22 1985 Nucleic Acids Research

Functional activity and chromatin configuration of SV40 enhancer injected in *Xenopus laevis* oocytes

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Received 5 August 1985; Revised and Accepted 24 October 1985

ABSTRACT

The SV40 enhancer microinjected into the nuclei of X. laevis oocytes is able to activate transcription about 100-fold when cloned upstream of the SV40 early promoter region or about 10-fold regardless of its orientation when located at a long distance from a test gene (bacterial chloramphenicol acetyl transferase). This effect is qualitatively and quantitatively similar to that observed when analogous constructions were transfected into mammalian cells. Making use of a direct labelling technique for the analysis of the chromatin structure we could show that the SV40 enhancer region in the microinjected plasmids is particularly accessible to cleavage with MNase or DNAse I. Single sites in the 72 bp repeats have been mapped at the nucleotide level.

INTRODUCTION

Enhancer elements are short DNA segments capable of activating in cis transcription from RNA polymerase II promoters (for reviews see 1 and 2). Peculiar properties of enhancers are their ability to potentiate initiation of transcription in an orientation-independent manner and to do this when located downstream or at a long distance from the coding sequence *in vivo* (3-7) or *in vitro* (8,9). Proximal promoters are activated at higher efficiency than distal ones (7) and the effect is exerted also on TATA-box elements deprived of their upsteam CAAT-box equivalent (7,9).

Since the discovery of viral enhancers many more sequences sharing the same properties have been identified in the genome of higher eukaryotes physically bound to genes whose transcription is cell or tissue-specific (10-15). These latter components of the enhancer family have been called, therefore, tissue-specific activators or enhancers. In general, viral

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enhancers also show a certain degree of species and tissue specificity (16,17); for instance, SV40 enhancer is more active in monkey cells than in murine cells, the reverse situation being true for the MSV enhancer (16,18). Nevertheless, viral enhancers can be considered universal gene activators whose effect is exerted, although at different levels, in many different eukaryotic cells.

Not much is known about the mechanism of gene activation by enhancer elements. It is universally accepted that enhancers bind one or more trans-acting factors (19), probably proteins, that are present in limiting concentrations in the nucleus. What happens after this interaction is not known in detail. It has been shown recently that the density of RNA pol. II molecules in close proximity to the SV40 enhancer is increased several fold (20,21), thus suggesting that these structures act as entry sites for the polymerase.

An important phenomenon associated with the presence of an active enhancer is a local perturbation of the chromatin structure. It is known that the SV40 enhancer is devoid of nucleosomes *in vivo* (22,23) and several DNAse I hypersensitive sites can be found in the SV40 and in the polyoma enhancer regions, whereas the adjacent promoters are resistant *in vivo* to this endonucleolytic cleavage (24-27). The same conformation of the SV40 enhancer was observed when this sequence was cloned into plasmids and introduced into mammalian cells with the DEAE-dextran technique (28,29). Moreover, it has been observed that the hypersensitive region is maintained in an SV40 mutant carrying a transposed enhancer (30) and is lost in a non functional SV40 enhancer mutant (6).

A suitable system to study the correlation between chromatin structure and DNA function is the frog oocyte. DNA microinjected into *X. laevis* oocyte nuclei is rapidly packed into chromatin (31,32). The function of the injected DNA can be easily tested because of the transcriptional activity of these cells (33).

In this paper we show that faithful transcription from SV40 early promoter is obtained in *X. laevis* oocytes when its own enhancer is present. On the other hand, two tissue-specific

enhancers are not functional in these cells. Microinjected SV40 enhancer sequences present micrococcal nuclease and DNAse I hypersensitive sites similar to those found in animal cells infected with SV40.

MATERIALS AND METHODS

Construction of plasmids

pSV2 CAT (34) and pA10 CAT-2 (16) were a gift from Prof. P. Gruss. pA10 CAT ENH3 and pA10 CAT ENH7: the SV40 enhancer early promoter region from the *Pvu*II (pos. 270) to the *Hind*III site (pos. 5171 (35) was first cloned into plasmid pUC8 (36) between the *Sma*I and the *Hind*III sites. The resulting recombinant, pUC-SV40-ENH was digested with *Eco*RI and *Nco*I and the 240 bp fragment containing the SV40 72 bp repeat and the 21 bp repeats was filled in with *E. coli* DNA pol. I, klenow fragment, and cloned into the *Bam*HI site of plasmid pA10 CAT-2. This had been previously linearized with *Bam*HI and the recessive 3' ends were filled in with nucleotides in presence of *E. coli* DNA pol. I, large fragment (B.R.L.). Two recombinants were obtained, called pA10 CAT ENH3 and pA10 CAT ENH7. Their structure was analyzed by restriction digestion. Both of them carry two copies of the insert in a head to tail arrangement. The complete insert is born in opposite orientations in ENH3 and ENH7 (Fig. 1).

Injection into X. laevis oocytes

Plasmid DNA was injected into stage V-VI oocytes at concentrations of 200-300 μg/ml (30 to 40 nl/oocyte). 25-30 oocytes/group were injected. After 24 hours of incubation in Barth medium at 21°C, oocytes were homogenized in Tris-HCl pH 8 using 10 μl/oocyte. CAT assays were done according to Gorman *et al*. (34).

S1 analysis and primer elongation

For the S1 analysis we used as a probe the *Bgl*II-*Hind*III fragment from plasmid pA10 CAT-2 (16), that goes from the SV40 *Sph*I site at position 133 to the *Hind*III site at position 5172. DNA was endlabelled at the 5' end with γ-^{32}P-ATP (Amersham Buchler) and T4 polynucleotide kinase (Bio Labs). Specific activity was 6×10^7 dpm/μg. 2×10^5 counts were used with RNA from two injected oocytes. Conditions for formation of DNA-RNA

hybrids and for digestion with S1 were as described (37). Primer elongation with a synthetic oligonucleotide complementary to the first 30 bases of the CAT mRNA was done as described (14).

Preparation of tagged DNA for chromatin studies

10 µg of pA10 CAT-2 and pA10 CAT ENH3 plasmid DNA was linearized with BamHI, the 5' phosphate groups were removed by treatment with alkaline phosphatase and the DNA was endlabelled with γ-^{32}P-ATP and T4 polynucleotide kinase. After 60 min incubation at 37°C the incubation mixture was chased for 60 min at 37°C with an excess of cold ATP (5 mM final concentration) and 10 units of polynucleotide kinase. The endlabelled DNA was diluted to 1-5 µg/ml and circularized in the presence of T4 DNA ligase (gift from Dr. V. Pirrotta). The ligation step was monitored on an agarose gel and the DNA then purified through Biogel columns (1 ml bed volume, 200-400 mesh, purchased from BIORAD) equilibrated with 5 mM NaCl, 5 mM Tris-HCl pH 7.5. The DNA was concentrated by ethanol precipitation.

Chromatin analysis of injected DNA

Tagged DNAs were dissolved in H_2O at a concentration of 200 µg/ml. In order to achieve optimal packaging in vivo into nucleosomes (38) carrier DNA (plasmid pEMBL8) was added to a final concentration of 1 µg/ml. 100-150 oocytes were injceted with each DNA; after 16-18 h incubation at 22°C, oocytes were gently homogenized in RSB (10 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl pH 7.5). For micrococcal nuclease analysis $CaCl_2$ was added to 1 mM final concentration and the homogenate was incubated at room temperature with 0.5 or 3.5 U of MNase (Sigma) /oocyte for different lengths of time. DNAse I digestions were performed by incubating the homogenates at room temperature with 20 ng/oocyte for 3 min. The nuclease digestions were stopped by addition of EDTA and SDS to final concentration 10 mM and 0.5% respectively, and the mixture was incubated with 500 µg/ml of proteinase K (Merck) for 1 h at 37°C. In both cases DNAs were extracted with phenol:chloroform and ethanol precipitated. After digestion with XbaI DNAs were electrophoresed on 1.2% agarose or on 6% denaturing polyacrylamide gels using a Tris-borate-EDTA system.

RESULTS

SV40 enhancer activates transcription in X. laevis oocytes

To test the activity of the SV40 enhancer in frog oocytes we used different fusions between SV40 early promoter, SV40 enhancer and the coding region of the bacterial gene for chloramphenicol acetyl transferase (CAT). These plasmids, whose structure is represented in Fig. 1, were microinjected into the nucleus of X. laevis oocytes. After 24 hours, oocytes were homogenized and the cytoplasmic fraction was tested for CAT enzymatic activity. The results are shown in Fig. 2. The most active template (pSV2CAT) carried the SV40 enhancer in its physiological location and orientation with respect to the SV40 early promoter. CAT activity is still abundant, although at a 10-fold lower level, when a duplicated SV40 enhancer is located downstream to the CAT coding region in both orientations (plasmids pA10 CAT ENH3 and pA10 CAT ENH7). A further 10-fold reduction is observed when only about 50 bp of one 72 bp element are left (pA10 CAT-2) (16). This response is identical to that observed when similar constructions carrying the SV40 enhancer cloned in various positions with respect to a test gene were assayed in mammalian cells (3-7).

We have determined the site of initiation of transcription in plasmid pSV2 CAT by S1 analysis. As a probe we used a Bgl2-HindIII fragment from plasmid pA10 CAT-2. This fragment covers the entire SV40 early region from the Sph1 site (pos. 133) to the HindIII site (pos. 5172) (35). The fragment was endlabelled

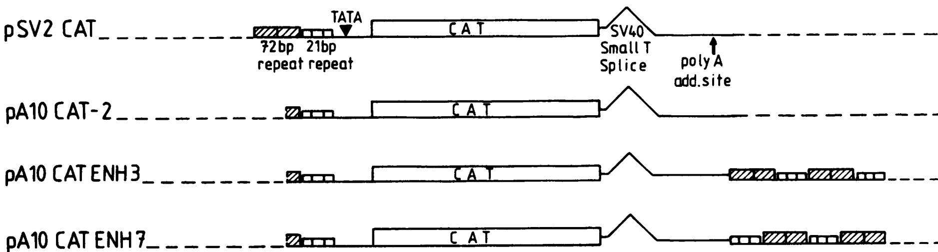

Figure 1: Schematic representation of SV40-CAT plasmids. Construction of the plasmids is described in the methods section. Hatched boxes represent SV40 enhancer 72 bp repeat sequences. Small boxes represent 21 bp repeats. Dotted lines represent pBR322 vector derived sequences.

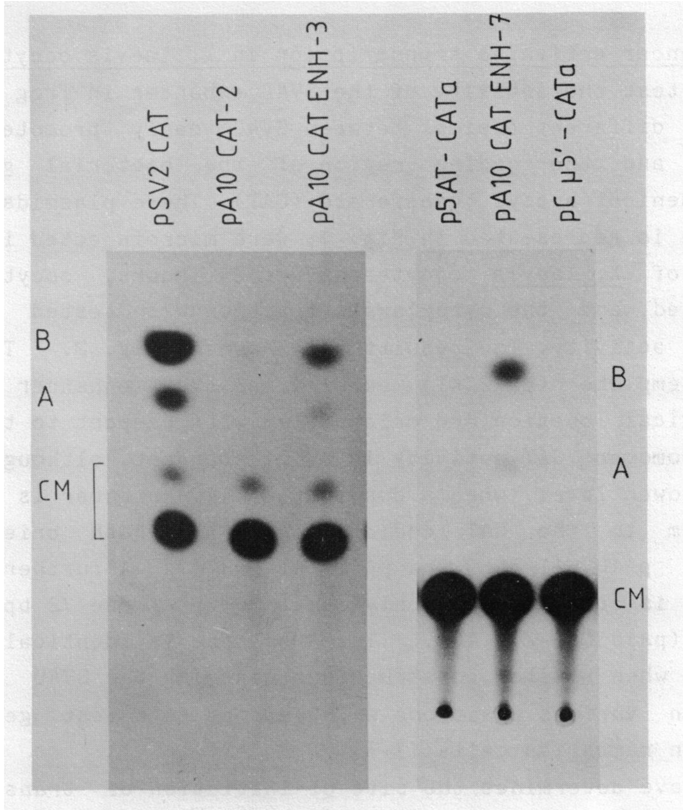

Figure 2: Expression of SV40 enhancer/SV40 promoter/CAT fusions in oocytes. Oocytes microinjected with plasmids carrying SV40 or tissue specific enhancers/CAT fusions were homogenized after 24 h incubation at $21^{\circ}C$. 50 µl of extract were used for CAT-assay as described in (34). p5'AT-CATa has been previously described (14): it carries the human α_1-antitrypsin 5' flanking region, about 1,200 bp long, cloned upstream of the SV40 promoter and in the "correct" orientation. pCµ5'-CATa carries the mouse Igµ enhancer (1 Kb, XbaI-XbaI fragment) cloned upstream of the SV40 promoter-CAT fusion (40). Chloramphenicol (CM); 1-Acetate chloramphenicol (A); 3-Acetate chloramphenicol (B).

with γ-^{32}P-ATP at the HindIII site. After annealing to total RNA extracted from oocytes microinjected with pSV2 CAT and S1 digestion, the protected fragments were separated on a polyacrylamide gel. The result is shown in Fig. 3, lane 2. Several protected fragments were obtained; the most abundant species are however, two groups of molecules (indicated by

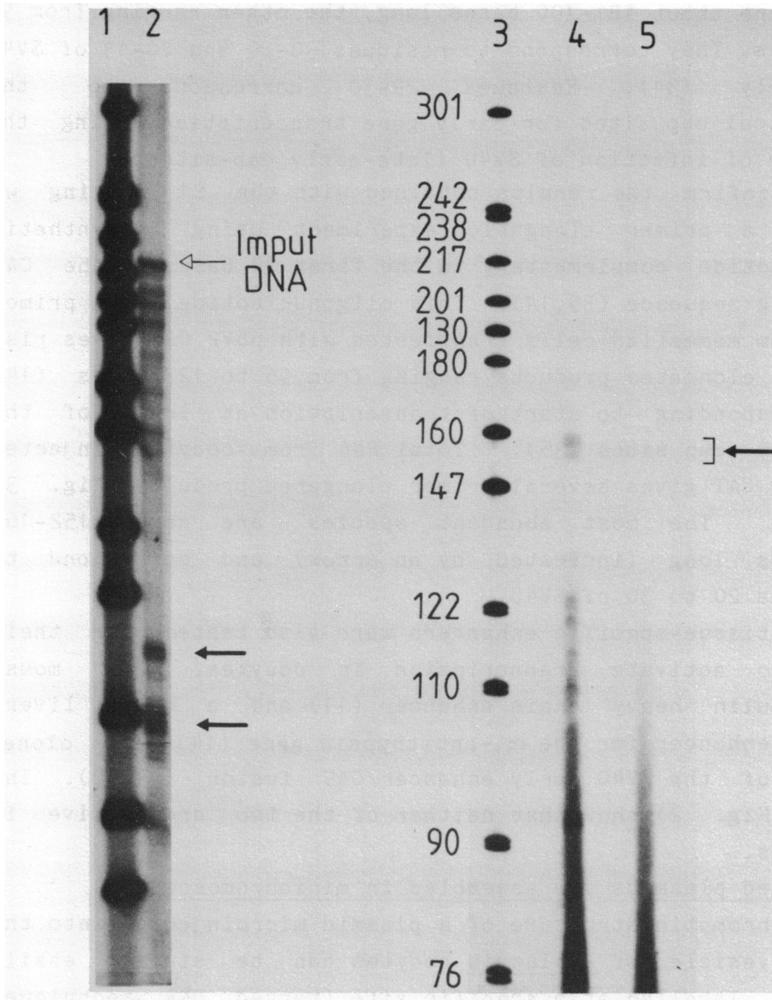

Figure 3: Determination of the transcription initiation sites from pSV2 CAT in X. laevis oocytes.
Lane 1: Endlabelled pBR322 digested with HpaII as size marker. Lane 2: RNA from 2 oocytes injected with pSV2 CAT was subjected to S1 analysis as described in the methods section. Black arrows indicate the major starts of transcription (see results section for description). Lane 3: Endlabelled pBR322 digested with HpaII. Lane 5: RNA from 5 uninjected oocytes and Lane 4: RNA from 5 oocytes injected with pSV2CAT was subjected to primer elongation using a synthetic oligonucleotide complementary to the first 30 bases of the CAT mRNA (14).

arrows), one about 101-100 bases long, the other ranging from 91 to 85 bases. They correspond to residues 30-29 and 20-13 of SV40 respectively (34). Residues 29-30 correspond to the physiological cap sites for early gene transcription during the late stage of infection of SV40 (late-early cap-sites).

To confirm the results obtained with the S1 mapping we performed a primer elongation experiment using a synthetic oligonucleotide complementary to the first 30 bases of the CAT mRNA coding sequence (39,14). This oligonucleotide, when primed on RNA from mammalian cells transfected with pSV2 CAT gives rise to primer elongated products ranging from 95 to 127 bases (14) and corresponding to start of transcription at level of the early-early cap sites (35). Total RNA from oocytes injected with pSV2 CAT gives several primer elongated products (Fig. 3, lane 5). The most abundant species are about 152-162 nucleotides long (indicated by an arrow) and correspond to nucleotides 20 to 30 of SV40.

Two tissue-specific enhancers were also tested for their ability to activate transcription in oocytes. A mouse immunoglobulin heavy chain enhancer (11) and a human liver-specific enhancer for the α_1-antitrypsin gene (14) were cloned upstream of the SV40 early enhancer/CAT fusion (14,40). The results (Fig. 2) show that neither of the two are active in these cells.

The injected plasmids are assembled in minichromosomes

The chromatin structure of a plasmid microinjected into the germinal vesicle of X. laevis oocytes can be studied easily using DNA labelled at a specific site (tagged DNA technique, 38). A circular plasmid is linearized with a restriction enzyme, labelled with γ-^{32}P-ATP after dephosphorylation with alkaline phosphatase, and recircularized with T4 DNA ligase. In the living oocytes the circular molecules acquire negative superhelical turns (37 and our unpublished observations) and are rapidly packed into chromatin. After a few hours of incubation, minichromosomes can be extracted and subjected to partial digestin with several different enzymes (MNase, DNAse I, etc.). DNA is then freed from proteins by phenol extraction and cut to completion with a different enzyme at an adjacent site. The main

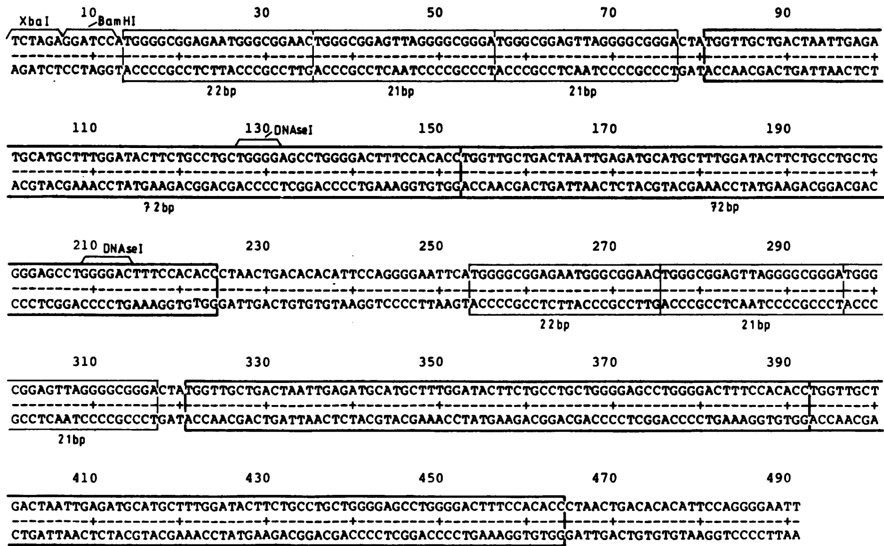

Figure 4: Sequence of the SV40 enhancer region in plasmid pA10 CAT ENH3. The sequence shown here starts from the adjacent XbaI and BamHI sites. The latter has been used for labelling the DNA, the former for recutting it after injection in the tagged DNA chromatin studies.

advantage of this technique is the possibility of mapping the nuclease cutting site(s) at the nucleotide level, if the two restriction endonuclease sites used for endlabelling and final cutting are sufficiently close to the region of interest.

Plasmid pA10 CAT ENH3 is an ideal substrate for the study of the chromatin structure assumed by the SV40 enhancer in the oocyte with the tagged DNA technique. This plasmid presents two unique restriction sites for BamHI and XbaI adjacent to the SV40 enhancer region (Fig. 4). DNA can therefore be restricted with BamHI, labelled at this site, ligated and microinjected. After MNase or DNAse I treatment the plasmid is digested with XbaI and electrophoresed. All the radioactivity comes from the region to the right of the XbaI site, i.e. the SV40 enhancers followed by the CAT coding region (3' towards 5'). As a control plasmid pA10 CAT-2 can be used. This has the same two restriction sites directly abutting the CAT coding region without interposed SV40 sequences.

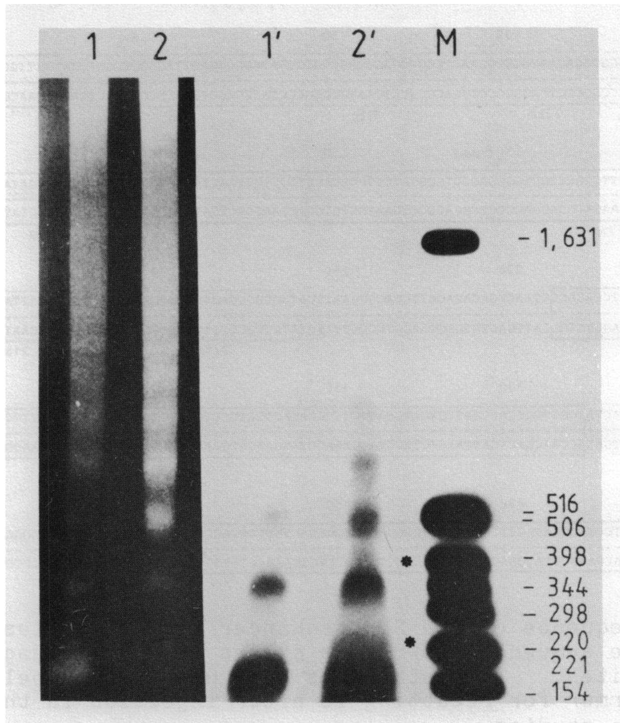

Figure 5: Nucleosomal pattern of DNA injected into X. laevis oocytes. Oocytes injected with ^{32}P-tagged pA10 CAT-2 or pA10 CAT ENH3 plasmids were homogenized and incubated with 3.6 units of MNase/oocyte for 18 min at room temperature and the material from 5 injected oocytes was electrophoresed on 1.5% agarose gel. Lanes 1 and 2: Ethidium bromide staining of DNA from pA10 CAT-2 injected oocytes (lane 1) or of DNA from pA10 CAT ENH injected oocytes (lane 2). Lanes 1' and 2' the same as for 1 and 2 but the gel was dried and autoradiographed at -80°C for 24 h.

In a preliminary experiment the two labelled injected plasmids were partially digested with MNase and the DNA electrophoresed on an agarose gel. The gel was stained with EtBr and then autoradiographed (Fig. 5). The staining reveals the nucleosomal pattern of X. laevis chromosomal DNA from oocytes and follicular cells together with that of the injected plasmid, but the label comes only from the injected DNA. At prolongued times of digestion, the pattern given by pA10 CAT ENH3 presents some irregularities with respect to the one obtained with pA10 CAT-2. One can see, between mono and dinucleosomes and between di- and trinucleosomes an extra band indicative of a region of

hypersensitivity to cleavage with MNase. Since the plasmids are labelled at the BamHI site, that is close to the enhancer region in pA10 CAT ENH3, the hypersenstive area must lie in this regulatory region.

Pattern of nucleosome distribution on the injected plasmids

To investigate further the chromatin conformation of the injected plasmids we mapped the MNase cleavage sites. With this aim, homogenized Xenopus oocytes injected with labelled pA10 CAT-2 and pA10 CAT ENH3 were separately incubated with MNase under conditions described in the legend to Fig. 6. The purified DNAs were restricted with XbaI and the double digestion products fractionated on a long agarose gel. We compared the pattern of MNase cleavage in the minichromosomes and in the corresponding naked DNA. Several conclusions can be drawn from the analysis of the autoradiogram (Fig. 6): The pattern of the naked pA10 CAT-2 plasmid (lane 8) is different from that given by the corresponding minichromosomes (lanes 6 and 7), thus suggesting that some of the preferential cleavage sites are protected by nucleosomal cores in the injected plasmid. On the other hand, the injected plasmid shows the appearance of new MNase cleavage sites (indicated with letters). Their distribution does not follow a regular 180 bp interval. These results are in favour of a non-random distribution of nucleosomes on the CAT coding region of plasmid pA10 CAT-2. These are probably distributed in more than two distinct phases.

Plasmid pA10 CAT ENH3 shows a similar pattern of cleavage. Also here many of the sites observed in the naked DNA disappear when the plasmid is covered by nucleosomes (compare lane 5 with lanes 3 and 4). In this case the CAT coding region is displaced, with respect to plasmid pA10 CAT-2 by about 500 bp, i.e. by the length of the duplicated SV40 enhancer/promoter region. The same cleavage sites are observed starting around nucleotide 700 (band A). This corresponds to the SV40 small T splice site that is cloned at the 3' end of the CAT coding region (33). The same band A is present around nucleotide 200 in plasmid pA10 CAT-2. Taking this site as a reference point and examining the CAT coding region in a 3' to 5' direction, the same pattern is obtained (from band B to band O). The first 500 bp of pA10 CAT

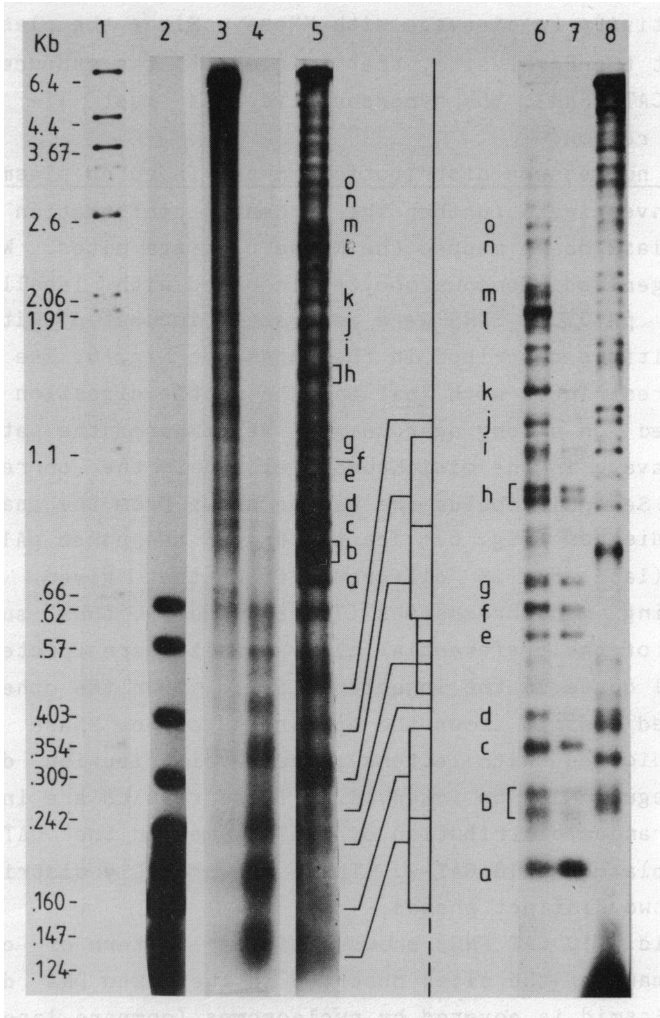

Figure 6: Mapping of micrococcal nuclease cleavage sites on assembled chromatin. X. laevis oocytes injected with labelled pA10 CAT-2 or with pA10 CAT ENH3 plasmids were homogenized and incubated with 0.5 U of MNase/oocyte for 7 and 15 min. Naked plasmids were also mildly digested with MNase. Purified DNAs were then restricted to completion with XbaI and the double digested DNA fragments fractionated onto a 1.2% agarose gel 40 cm long. The gel was dried and autoradiographed.
Lane 1: Endlabelled λDNA digested with ClaI; lane 2: Endlabelled pBR322 digested with HpaII; lane 3 and 4: Naked pA10 CAT ENH3 digested at 0°C with 0.1 U of MNase/10 μg of DNA for 3 and 10 min respectively; lane 5: Injected pA10 CAT ENH3 digested with MNase for 7 min; lane 6 and 7: Injected pA10 CAT-2 digested with MNase for 7 and 15 min respectively; lane 8:

Naked pA10 CAT-2 digested with MNase for 6 min in the same conditions as for pA10 CAT ENH3. Letters from a to o indicate bands in common between equivalent regions of the two injected plasmids.

ENH3 starting from the *XbaI* site are constituted by the duplication of the SV40 early promoter/enhancer (schematically drawn in the middle of Fig. 6). Here, several new preferential cleavage sites are observed in the minichromosome together with those present in the naked DNA. They correspond to specific segments of the four 72 bp elements and to the interposed 21 bp repeats. This finding favours the assumption that this region is devoid of nucleosomes in *X. laevis* oocyte nuclei.

DNAse I hypersensitive sites are located in the enhancer

In the previous section we showed that the promoter/enhancer region of the minichromosomes assembled in living frog oocytes exhibits a pattern of hypersensitivity to MNase digestion. To investigate further the chromatin structure of this region we decided to treat the pA10 CAT ENH3 plasmid with DNAse I after microinjection in order to compare the pattern of sensitivity to this enzyme with that obtained in mammalian cells. Naked and injected pA10 CAT ENH3 were therefore subjected to limited digestion with DNAse I and then fully linearized with *XbaI*. Low molecular weight DNA species were resolved on a 10% polyacrylamide gel. The results are shown in Fig. 7. Only in lane 2 (injected DNA) is there a clearly evident, very intense DNAse I cut which maps about 130 bp from the *XbaI* site, that is, in the SV40 enhancer region (see Fig. 5). This region is not hypersenstive in the naked DNA. In the equivalent position of the second 72 bp repeat, between 210 and 215 bp from the *XbaI* site (Fig. 4) a faint band can be seen. It probably constitutes an analogous, although less exposed, DNAse I hypersensitive area. However, the presence of a smearing of radioactivity in this part of the gel does not allow us to draw a firm conclusion about this point. Several other faint bands are also present at a longer distance from the *XbaI* site. Their precise mapping was not possible, owing to the lower power of resolution in this part of the gel and to the smearing of the radioactivity.

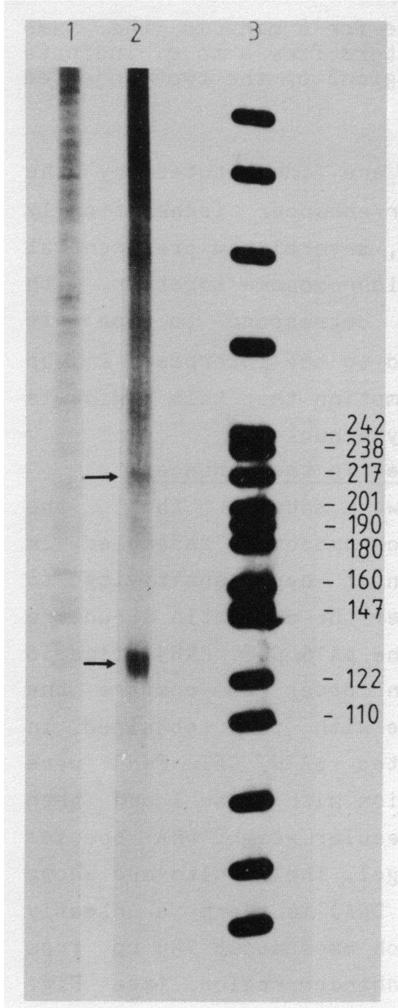

Figure 7: Mapping of DNAse I hypersensitive sites in the enhancer region. Naked and assembled tagged pA10 CAT ENH3 plasmid were mildly digested with DNAse I followed by XbaI treatment. The products were displayed on 6% non denaturing polyacrylamide gel.
Lane 1: Naked DNA incubated at room temperature with 1 ng of DNAse I/14 µg of cold vector DNA for 3 min; lane 2: Injected DNA incubated with 20 ng of DNAse I/oocyte for 3 min; lane 3: pBR322 digested with HpaII. Arrows point to the DNAse I hypersensitive sites observed in the injected plasmid.

DISCUSSION

In this paper we show that the enhancer from SV40 virus is able to stimulate transcription from the adjacent SV40 region at high efficiency when microinjected into living *X. laevis* oocytes. The SV40 enhancer stimulates promotion of transcription in Xenopus oocytes from the late-early cap sites of the homologous early promoter and from other non physiological starts in the same region. The effect is observed when the enhancer is cloned upstream or downstream from the CAT gene and is orientation independent. This finding is similar to

what has been observed with analogous constructions transfected into mammalian cells (3-7).

It has been speculated that the activity of the SV40 enhancer depends on the binding of a proteic trans-acting factor present in limiting amounts in the cell nucleus (3-7). From our experiments we hypothesize that a structurally analogous factor must exist in Xenopus oocytes. The physiological role of this (or these) protein(s) is not known. One might speculate that it is somehow involved in the expression of endogenous genes during oogenesis or embryogenesis. If the same enhancer (SV40) is functional at high efficiency in man, rodents and amphibians, the protein responsible for its recognition must be well conserved during evolution.

In agreement with the previous experiments in mammalian cells, tissue-specific activators are not functional in oocytes. Also in Xenopus laevis therefore, differential expression in different tissues is probably due to the production of different classes of trans-acting factors each one capable of recognizing only a subset of tissue-specific activators; all classes are however able to bind and activate "universal" enhancers like the SV40 enhancer.

The analysis of the chromatin structure of the SV40 enhancer in frog oocytes reveals several interesting features:

a) The enhancer region itself is a region hypersensitive to digestion with MNase or DNAse I, thus suggesting that it is present in a naked configuration.

b) Some of the DNAse I sites have been mapped at the nucleotide level. This has been possible by making use of the tagged DNA technique (38). These sites more or less correspond to sites that have been detected in mammalian cells, when a transfected DNA carrying a functional SV40 enhancer was used that was not able to replicate (28). It is also known, in fact, that plasmids microinjected into X. laevis oocyte nuclei do not undergo active DNA replication (41).

c) The enhancer does not influence the nucleosomal distribution in the adjacent DNA.

The coordinated study of function and chromatin structure is particularly feasible on DNA microinjected into X. laevis

oocytes. Our approach could turn out to be very useful for the fine dissection of the steps that lead to gene activation. Frog oocytes are in fact optimal substrates for in vivo complementation studies in which promoters or enhancers not functional or not presenting a peculiar chromatin conformation, acquire these characteristics following coinjection of transcriptional factors extracted from nuclei where the same enhancers or promoters are physiologically active.

ACKNOWLEDGEMENTS

We are grateful to Prof. R. Cortese for helpful discussion and continuous encouragement and to J. Hughes for revising the manuscript. We also thank Dr. G. Paonessa for help in the microinjections, Petra Stevenson for technical assistance and Ines Benner for typing the manuscript. The work done in Italy was supported by the "Progetto Finalizzato CNR Ingegneria Genetica e Basi Molecolari delle Malattie Ereditarie". G.S. stay at the EMBL was suported by a grant of the "Ministero della Pubblica Istruzione" to Prof. F. Salvatore.

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