TRANSCRIPTION OF SV 40 NUCLEOPROTEIN COMPLEXES IN VITRO

C. Crémisi[1], A. Chestier[1], C. Dauguet[2], and M. Yaniv[1]

[1] Department of Molecular Biology and
[2] Department of Virology,
Institut Pasteur, 75015 Paris (France)

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Abstract.

SV 40 minichromosomes extracted from nuclei of infected cells with an average of 21 nucleosomes or minichromosomes partially or totally deproteinized by treatment with increasing concentrations of KCl were transcribed with the E.coli RNA polymerase. Removal of nucleosome resulted in a marked stimulation of the ^{3}H-UTP incorporation. Analysi of the RNA synthesized in vitro by sucrose gradient sedimentation revealed that the transcription products of native complexes are relatively short sedimenting between 3 S - 10 S, whereas the RNA transcribed from KCl treated complexes is much longer with a considerable fractior sedimenting faster than 16 S. Hybridization of RNA fractions along the gradients proved that the size of the viral specific RNA increased after the removal of the nucleosomes from the template. These results suggest that under these conditions the nucleosomes (or a factor removed by KCl) block the elongation of RNA chains in vitro.

Introduction.

The basic structural features of the chromatin fibre have been es tablished in the last three years[1,2,3,4,5,6,7]. Repeating units - nucleosomes or nu bodies- formed by the association of 160 to 200 base pairs of DNA with 8 molecules of histone (H2A,H2B,H3 and H4) are inter connected by short segments of DNA. It would be interesting to determine whether the tight association existing between histones and DNA i maintained in actively transcribing regions. Several workers have attempted to resolve this problem but the results have been conflicting. The electron microscopy studies of Franke et al.[8] demonstrate that t active genes of ribosomal DNA do not conserve the nucleosomic structur Other studies on non ribosomal[9,10] and ribosomal[11] genes seem to suggest that the active genes are likely to be packages by histones, but that these nucleosome structures are in some way modified, since i has been found that the associated DNA is extremely sensitive to diges tion by pancreatic deoxyribonuclease I. Several studies which have bee carried out on SV 40 and papovavirus chromatins, which are known to ha ve a similar structure to cellular chromatin[12,13,14] have suggested that the viral DNA may be associated with histones during its replica-

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tion[13,15,16,17] and transcription[18]. However our observations on the stability and the absence of mobility of the viral nucleosomal structure at physiological ionic conditions *in vitro*[19] and the absence of fast nucleosomal exchange below 880 mM ammonium sulfate[20] raise difficulties concerning the transcription of the viral chromatin. In an attempt to clarify the fate of nucleosomes during gene expression, we studied the transcription of these complexes *in vitro* with the *E.coli* RNA polymerase, and our results suggest that the nucleosomes block the elongation of RNA chains *in vitro*.

Materials and Methods.

Preparation of nucleoprotein complexes (NPC) : African green monkey kidney cells (CV1) were infected at a multiplicity of 50 PFU/cell with a plaque-purified wild type strain of SV 40 and labeled with C^{14} thymidine at 32 hours post infection. At 42 hours, the cells were collected, the nuclei were isolated and the nucleoprotein complexes extracted as previously described[13].

Transcription with E.coli RNA polymerase : *E.coli* RNA polymerase was a gift of B.Lescure and S.Saragosti[21], the enzyme was purified from *E.coli* MRE 600, as described by Humphries[22] and contained 0.85 to 0.90 equivalent of sigma, as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. The NPC were first incubated at 37°C in the presence of increasing concentrations of salt 0.2 M, 0.5 M or 1M (0.2 M NaCl supplemented with KCl) after 30 min the ionic strength was adjusted to 0.2 M KCl by dilution and the different samples were incubated for 30 min. at 37°C in a standard transcription system containing : approximately 1 µg/ml viral DNA, 30 mM Tris HCl (pH 7.9), 10 mM $MgCl_2$, 150 mM KCl 0.1 mM dithiothreitol, 0.2 mM CTP, ATP, GTP, 0.04 mM UTP, 70 µCi H^3 UTP (50 Ci/mmole) and 10,5 units of *E.coli* RNA polymerase in a final volume of 350 µl. The reaction was initiated by the addition of the UTP which had been previously omitted for two minutes. After intervals of 10, 20 and 30 min., 35 µl aliquots were taken, precipitated for 15 min. with 5% cold trichloroacetic acid containing 0.05 mM pyrophosphate and collected on nitrocellulose filters. The radioactivity on dried filters was monitored by liquid scintillation counting.

Purification of RNA and sedimentation analysis: In some cases, the synthesis of RNA was terminated by treating the reaction mixture for 50 min. at 37°C with 40 µg/ml of RNAse free DNAse, followed by the addition of EDTA and SDS to a final concentration of 20 mM and 0.5% (w/v) respectively, otherwise the EDTA and SDS were added directly, and the resulting solution was passed through a sephadex G50 column. The fractions containing the RNA were pooled and extracted with an equal volume of Tris saturated phenol. The aqueous phase was reextracted twice with an equal volume of chloroform and precipitated with two volumes absolute ethanol at -20°C. After centrifugation, the precipitate was dissolved in 10 mM Tris (pH 7.9) 1 mM EDTA and heat denatured for 1 min. at 100°C followed by quick cooling to 0°C before it was layered on a 3.8 ml gradient of 5-20% sucrose containing 0.1% SDS, 200 mM NaCl, 10 mM Tris HCl (pH 7.4) 1 mM EDTA. The RNA from different parts of the gradient was pooled and precipitated with 2 volumes absolute ethanol at -20°C centrifuged, dissolved in 10 mM Tris HCl (pH 7.9), 1 mM EDTA and used for DNA-RNA hybridization.

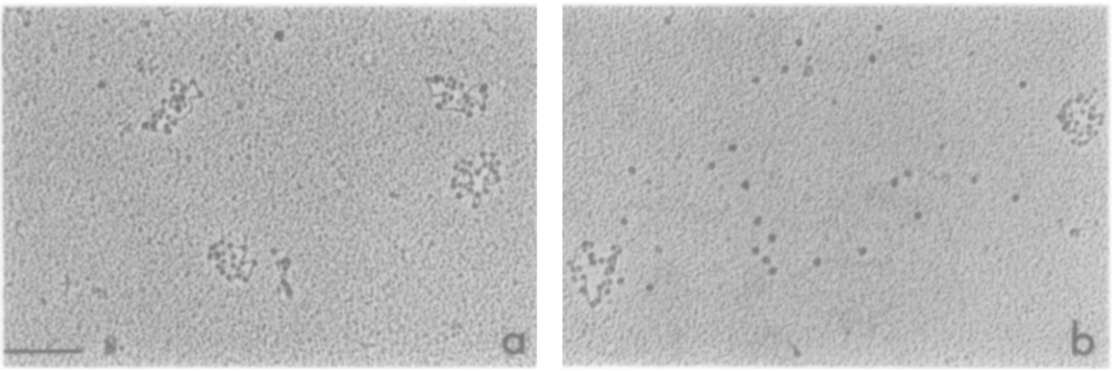

Figure 1. Electron micrographs of SV 40 nucleoprotein complexes. (a) complexes extracted from SV 40 infected cells at 0.2 M NaCl ; (b) same complexes after transcription. Samples were prepared for electron microscopy according to Dubochet technique (see 13) and rotary shadowed with Pt-Pd as previously described. Bar : 200 nm.

DNA-RNA hybridization : DNA-RNA hybridization was carried out according to the method described by Kourilsky and coll.[23] in 2xSSC 40 redistilled formamide for 48 hours at 42°C (optimal conditions) using excess SV 40 DNA (0.6 µg/filter). The purified SV 40 DNA was alkali denatured then heat denatured and finally irreversibly fixed to nitrocellulose filters. After incubation, the filters were deeped in chloroform, washed 3 times with 2xSSC, then treated with preboiled RNAse (20 µg/ml in 2xSSC, 45 min. at 20°C), washed 5 times with 2xSSC, dried and counted in toluene base scintillation liquid. Each RNA sample was also hybridized to filters containing mouse DNA and the background subtracted from the values obtained with viral DNA.

Results.

SV 40 minichromosomes were selectively extracted from infected nuclei in the presence of 0.2 M NaCl and 0.25% Triton X-100 and further purified by sedimentation (S value 55 S) on neutral sucrose gradients. Native DNA-histone complexes contained on average 21 ± 2 nucleosomes as verified by electron microscopy (Fig. 1a). The ratio of proteins to DNA in these complexes is approximately 1:1[13,16,17]. Polyacrylamide gels analysis showed the presence of the four histones, trace of histone H1 and low contamination with cellular and viral proteins (VP1). No cellular DNA or chromatin contaminated these complexes (results not shown). Partial or total removal of nucleosomes was achieved by incubating aliquots in the presence of 0.5 M or 1 M salt for 30 min. at 37°C followed by rapid dilution to a final salf concentration of 0.2 M KCl. Elec

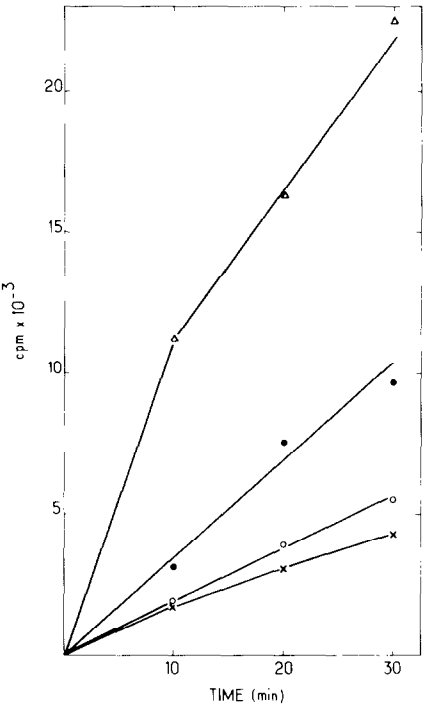

Figure 2. Kinetics of transcription of NPC in vitro with E.coli polymerase . NPC treated at different ionic strength was transcribed in vitro with E.coli polymerase. (x——x) activity of RNA polymerase alone ; (o——o) transcription of NPC 0.2 M ; (●——●) transcription of NPC pretreated 30 min at 0.5 M KCl ; (Δ——Δ) transcription of NPC pretreated 30 min at 1 M KCl. The values given represent the total incorporation.

tron microscopy examination of these samples showed that the 0.5M salt treated material contained a decreased number of nucleosomes (S value 35 S) per DNA molecule, the 1 M treated material appears as naked super helical which sedimented on neutral sucrose gradients as deproteinized viral DNA (S value 21 S). These results agree with previous sedimentation studies on SV 40 and polyoma minichromosomes[24]. The rapid dilution of the samples and their low DNA concentrations does not enable the reconstituation of nucleosomes[7]. Native or salt treated complexes were diluted to identical final DNA concentrations and transcribed with excess E.coli RNA polymerase in the presence of 0.15 M KCl. As shown in Fig. 2, removal of nucleosomes results in a marked stimulation of the H^3 UMP incorporation. The stimulation varied from 4 to 20 fold for 1 M KCl treated complexes in different experiments. Increase in the concen-

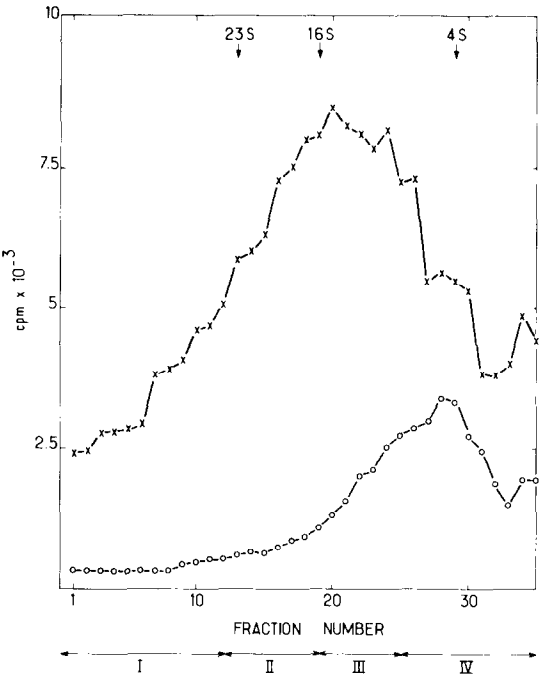

Figure 3. Sedimentation analysis of RNA synthesized by NPC. RNA was prepared as described in Materials and Methods. Samples (150 ul) were layered onto 3.8 ml 5-20% sucrose gradient containing 0.1 % SDS and sedimented 2 h 15 at 55.000 rpm (15°) in a SW 56 rotor. (o——o) RNA synthesized by NPC 0.2 M ; (x——x) RNA synthesized by NPC treated at 1 M KCl.

tration of the viral complexes beyond 1 µg/ml of DNA resulted in a decrease of the stimulation observed after the 1 M KCl treatment. Probably the presence of higher concentrations of free histones and DNA in the transcription mixture resulted in random binding of the histones t the DNA without formation of nucleosomes. It was shown that addition c histones to polyoma DNA markedly inhibits its template activity[25]. We noted that 50 to 80% of H^3 UMP incorporated with template maintaine at 0.2 M NaCl are template independent products. In fact, it was repor ted that the E.coli RNA polymerase is capable to incorporate ribotriphosphate in absence of DNA[26]. Due to the low efficiency of the mini chromosome as a template for transcription we have been forced to use saturating amount of enzyme (10 units) in each reaction mixture, this explains the high percentage of template independent incorporation we find in this system. When the appropriate amount of free DNA and limi-

Table I. Hybridization of *in vitro* cRNA to SV40 DNA. The gradient was divided into four parts : part I - fraction 1 to 12 ; part II - fraction 13 to 19 ; part III - fraction 20 to 25 ; part IV - fraction 26 to 35. The RNA from different parts of the gradient was pooled and precipitated with 2 volumes absolute ethanol at -20°C centrifuged, dissolved in 10 mM Tris HCl (pH 7.9), 1 mM EDTA and used for DNA-RNA hybridization. Hybridization results are a mean of two determinations. * RNA was heated during 1 min. at 100°C before hybridization.

Nature of template	RNA fractions	I	II	III	IV
0.2 M KCl treated complex	RNA per fraction (%)	8	11	27	55
	Hybridized RNA (%)	15	23	23	8
	Percentage of total viral specific RNA	8	18	44	30
1 M KCl treated complex	RNA per fraction (%)	23	28	26	23
	Hybridized RNA (%)	50(75)*	78(100)*	39(58)*	26(49)*
	Percentage of total viral specific RNA	16(24)*	31(41)*	14(21)*	8(14)*

ting enzyme are used, the template independent incorporation is lower than 1% of the total counts.

Electron microscopy examination of the viral complexes after transcription did not reveal any modification in the number of nucleosomes nor any evident clustering of the nucleosomes. Since the size of the RNA polymerase molecules observed on the grids is very similar to that of the nucleosomes, we cannot distinguish enzyme bound on the viral minichromosomes (Fig. 1b).

RNA transcribed from the native or 1 M KCl treated complexes was purified and analysed by sedimentation on sucrose gradients. As shown in Fig. 3, the transcription products of native complexes are relatively short sedimenting between 3 - 10 S, whereas the RNA transcribed from 1 M KCl treated complexes is much longer, with a considerable fraction sedimenting faster than 16 S. The size of the template independent products was found to be extremely short, sedimenting at about 4 S (data not shown). The RNA gradients were subdivided into the four fractions indicated in Fig. 3, the RNA pooled, concentrated by ethanol precipitated and then hybridized to SV 40 DNA immobilized on nitrocellulose filters. Several series of experiments were performed and one of them is presented in Table I, showing that more than 50% of the RNA

transcribed from 1 M KCl treated complexes hybridized to SV 40 DNA. Higher percentage of hybridization is observed in fractions containing longer RNA chains (fractions I and II). On the contrary, only low degree of hybridization is observed with RNA transcribed from native complexes and the bulk of the viral specific RNA is contained in the slow sedimenting fractions III and IV (Table I). However, we must recall for this last case that 50-80 per cent of cpm correspond to the template independent products made by the E.coli RNA polymerase and that most of these products sediment in the fourth fraction. These results suggest that nucleosomes block the elongation of RNA chains initiated on the native complexes. To examine an eventual role of histone H1 in nucleosomes mobility, we tried to transcribe the viral complexes in the presence of calf thymus histone H1 (1 γ/ml) and observed an inhibition of 30%. Transcription of purified viral DNA was inhibited by 14% under similar conditions. The native complexes became more condensed after addition of H1 when examined by electron microscopy in agreement with the observations of Germond et al.[27].

Discussion.

Contrary to the behaviour of cellular chromatin, the viral chromatin remains in solution during the transcription and the fate of the template can be easily followed by electron microscopy. As we have previously shown, the 21 nucleosomes of the viral minichromosome are not mobile and are located at random on the viral genome[19]. It is known that the strong promoter site for E.coli RNA polymerase is localised a position 0.17 on SV 40 DNA[28,29,30], in addition in the presence of excess enzyme, several additional initiation sites are detected on superhelical or linear SV 40 (Saragosti, unpublished). It is probable therefore that only a fraction of the minichromosomes would contain th strong promoter or any other initiation sites in an accessible position. The observed decreased template activity of the minichromosomes relative to KCl treated complexes can be due to limitation of initiations only or additionnally, to restriction of the elongation by the nucleosomes. The size measurements of the virus specific RNA clearly demonstrates that the nucleosomes restrict also the elongation of the in vitro transcription products. Mainly short RNA chains ranging between 3 S and 10 S are synthesized from the native complex whereas RNA chains up to full genome length are synthesized from 1 M treated complexes devoid of nucleosomes. Since both transcription mixtures contained initially the same amount of purified complex, the preferential

degradation of the RNA made from native complexes is unlikely. The process of RNA synthesis is not sufficient to dislocate the DNA relatively to the histones core and thus permits chain elongation.

Huang et al.[31] have previously transcribed *in vitro* nucleoprotein complexes extracted from SV 40 virus by alkali dissociation. In fact, it is now well known[32,33] that this procedure does not preserve the integrity of the nucleoprotein complex. A fraction of histones is lost during extraction. Nevertheless, our results are in accord with those of Huang et al. - the efficiency of transcription *in vitro* increases after deproteinization of the template. They obtained also similar results with mammalian RNA polymerase II (B) isolated from SV 40 infected monkey cells : naked DNA was sevenfold more efficient than DNA histones complexes.

Green et al.[18] isolated viral transcription complexes containing endogeneous RNA polymerase from SV 40 infected cells in the presence of 0.4 M NaCl. From their sedimentation profiled it is reasonable to suppose that the DNA in these complexes is still associated with histones (under these ionic conditions, we did not observe any removal of nucleosomes). The low level of endogeneous activity can be due only to the short elongation of the nascent RNA chains. No size measurements were made with these products. This low endogeneous transcription activity is stimulated by addition of sarkosyl[34] that was shown previously to remove the histones from the DNA without inactivating the preinitiated RNA polymerase[35]. These experiments suggest that even the *in vivo* initiated RNA polymerase elongates poorly *in vitro* in presence of nucleosomes. The process of viral genome transcription *in vivo* (initiation and elongation) may require continuous nucleosome modifications comparable to the modified state of nucleosomes in active genes[9,10].

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