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Biochemistry

Minichromosome from BK virus as a template for transcription *in vitro*

(viral chromatin/nucleosomes/blot-hybridization)

GUERRINO MENEGUZZI*, PIER FRANCO PIGNATTI†, GIUSEPPE BARBANTI-BRODANO‡, AND GABRIELE MILANESI*†

* Centre de Biochimie, Faculté des Sciences, Nice, France; † Laboratorio di Genetica Biochimica ed Evoluzionistica del Consiglio Nazionale delle Ricerche, Istituto di Genetica, Pavia, Italy; and ‡ Istituto di Microbiologia, Università di Ferrara, Italy

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ABSTRACT **BK virus DNA can be extracted from virions as a nucleoprotein complex containing about 20 nucleosomes. Transcription of this "minichromosome" with *Escherichia coli* RNA polymerase indicates that both initiation and elongation of RNA chains are reduced by the presence of nucleosomes. Hybridization analysis of RNA made on the complex shows preferential transcription of one region of BK virus genome. No increase in strand selection is observed with respect to transcription of purified superhelical BK virus DNA.**

In recent years, a number of studies have been reported on the structural and biological properties of human papovavirus BK (1–5). Originally isolated from the urine of an immunosuppressed patient (6), this virus has been shown recently to induce malignant transformation in hamster cells (7–9). Interest in this infectious agent has further increased since it was shown that antibodies to BK virus (BKV) are widespread in the human population (10–12) and since BKV DNA was found in human tumors (13).

Biochemical studies of the BKV infective cycle in cultivated cells are made difficult by the relatively low efficiency of infection in systems used so far (14). Nothing is known, for example, about BKV DNA transcription in productively infected or transformed cells.

Several groups of investigators have been able to demonstrate a chromatin-like organization of polyoma and simian virus 40 (SV40) DNA in infected cell nuclei (15–18). Repeating units in which 8 histone molecules of cellular origin are associated with approximately 200 base pairs of DNA have been described by electron microscopic and biochemical techniques. Practically all the viral DNA in infected cells was found to be associated with histones (16, 19), to give an average of 21 randomly distributed repeating units (called nucleosomes or ν-bodies). Therefore, in studies of viral transcription, the possibility must be considered that this "minichromosome" (15) form of papovavirus DNA (rather than naked DNA) is the transcription template.

Virus disruption at alkaline pH also yelds nucleoprotein complexes of SV40 DNA (20–22). Recently, Christiansen *et al.* (18) have reported that treatment at pH 9.8 liberates a DNA–histone complex from SV40 virions that is indistinguishable from the minichromosome seen in infected cells. We report here that BKV DNA is also organized as a minichromosome within the virions and that this complex can be transcribed by *Escherichia coli* RNA polymerase. The RNA synthesized is compared to that obtained with deproteinized BKV DNA.

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MATERIALS AND METHODS

Virus Growth and Purification. BKV was grown in Vero cells and purified by centrifugation in KBr saturated solution, followed by two cycles of centrifugation in CsCl, as described (14).

Virus Disruption and Complex Isolation. The method of Christiansen *et al.* (18) was followed with minor modifications. Fifty microliters of virus suspension (containing 15 μg of viral DNA) was warmed at 37° in the presence of 2 mM dithiothreitol. After 5 min, an equal volume of 0.2 M glycine (pH 9.85) was added and the incubation was continued for 5 min. The sample was then chilled and layered on top of a 4.8-ml 5–20% sucrose gradient, containing 0.1 M NaCl, 10 mM Tris·HCl (pH 7.5), 1 mM EDTA, and 0.25% Triton X-100 (SERVA). Gradients were spun at 45,000 rpm for 75 min in a Spinco SW50.1 rotor at 4° and 200-μl fractions collected. Purified BKV DNA was run as a marker in a separate tube.

Electron Microscopy. Samples from sucrose gradient fractions were prepared for electron microscopy (without prior fixation) according to the method of Dubochet *et al.* (23) as described (16).

DNA Purification. BKV DNA was extracted from purified virions as follows. Five milliliters of virus suspension containing 10 mM Tris·HCl (pH 7.5), 10 mM EDTA, 0.5% sodium dodecyl sulfate ($NaDodSO_4$), and 50 μg of proteinase K per ml (Merck) was heated at 50° for 30 min and then incubated at 37° for 3 hr. CsCl (5.3 g) and ethidium bromide (2 mg) were then added and the density was adjusted to 1.600 g/cm^3. After centrifugation in a Spinco Ti50 rotor at 43,000 rpm and 10° for 50–60 hr, fractions containing the superhelical form (F I) of DNA were pooled, extracted with isopropanol, and dialyzed against 10 mM Tris·HCl (pH 7.5)/1 mM EDTA.

Transcription Assay. Fractions from the sucrose gradient used to isolate the nucleoprotein complex (see above) were assayed for template activity in an *in vitro* transcription system with *E. coli* RNA polymerase. Assays were performed by adding 50 μl of each fraction to 50 μl containing 80 mM Tris·HCl (pH 7.9), 20 mM $MgCl_2$, 200 mM NaCl, 0.8 mM EDTA, 8 mM dithiothreitol, 0.8 mM each ATP, GTP, and CTP, and 0.4 mM [α-^{32}P]UTP (specific activity 8×10^4 cpm/nmol). Final NaCl concentration was 150 mM. Reactions were started by addition of 5 μl of *E. coli* RNA polymerase (0.5 unit, purification procedure to be described elsewhere). After a 30-min incubation at 37°, reactions were precipitated with 5% trichloroacetic acid/1% Na pyrophosphate and filtered on GF/C filters. Radioactivity was then determined. In all experiments,

Abbreviations: BKV, BK virus; SV40, simian virus 40; $NaDodSO_4$, sodium dodecyl sulfate.

RNA synthesis was totally dependent on added RNA polymerase.

When RNA was to be purified for further analysis, aliquots from the peak fractions of the gradient were pooled and the size of the reaction mixture was increased accordingly. Specific activity of radioactive UTP (labeled with ^3H or ^{32}P) was also increased, as indicated for each experiment. In a parallel reaction, equivalent amounts of purified BKV DNA, sucrose, Tris, NaCl, EDTA, and Triton X-100 were added instead of the gradient pool. After 30 min at 37° $NaDodSO_4$ and EDTA were added to 1% and 20 mM, respectively. RNA was then purified by Sephadex G-75 chromatography, phenol/chloroform extraction, and ethanol precipitation.

Sedimentation Analysis. Band sedimentation was performed through 5–20% sucrose gradients in 80% formamide, 10 mM Tris·HCl (pH 7.9), 2 mM EDTA, and 0.2% $NaDodSO_4$. RNA samples (5–10 μl) were added to 70 μl of gradient solution, (without sucrose), heated at 65° for 6 min, layered on top of gradients, and spun for 380 min at 58,000 rpm and 24° in a Spinco SW60Ti rotor.

Self-Complementarity Assay. RNA preparations were digested with electrophoretically purified DNase (Merck) that had been treated with iodoacetate (24). After phenol/chloroform extraction and ethanol precipitation, RNA was incubated at 70° in 0.3 M NaCl/30 mM Na citrate and RNase resistance was determined as described (25).

Hybridization to BKV DNA Restriction Fragments. BKV DNA was digested with *Hin*dIII endonuclease (Miles) as described by Howley *et al.* (5). Fragments were separated by electrophoresis in 1% agarose (BioRad), transferred to nitrocellulose membranes, and hybridized to BKV [^{32}P]RNA as described by Southern (26).

RESULTS

Isolation and electron microscopic visualization of BKV minichromosome

Exposure of SV40 particles to high pH results in virion disruption and liberation of DNA–protein complexes (20). Huang *et al.* (21) have shown that treatment at pH 10.5 yelds a complex sedimenting at 35–40 S in sucrose gradients. More recently, Christiansen *et al.* found that exposure at pH 9.8 liberates a 60S DNA–histone complex, closely resembling viral minichromosomes isolated from SV40-infected cells (18).

When purified BKV particles were treated at pH 9.8 at 37° for 5 min and then centrifuged in a neutral sucrose gradient, most of the material sedimented in a rather broad peak, with an average sedimentation coefficient of 55–60 S (Fig. 1). Peak fractions were then examined by electron microscopy, without prior fixation, by the technique of Dubochet *et al.* (23). As shown in Fig. 2, the 55–60S material was composed of closed molecules in which beaded structures (nucleosomes) were connected by short filaments (DNA) of variable length. Such complexes are very similar to the minichromosomes obtained from SV40 virions (18) or virus-infected cells (15, 16). The average number of nucleosomes per complex was 20 in fraction 11. No "naked" DNA was seen in any of the gradient fractions. Material sedimenting in the small peak around fraction 20 appeared as small granular structures, presumably composed of aggregated capsid proteins.

Template activity of isolated complex

Fractions from the sucrose gradient were directly assayed for template activity with *E. coli* RNA polymerase. Incorporation values followed the absorbance profile (Fig. 1), with the top

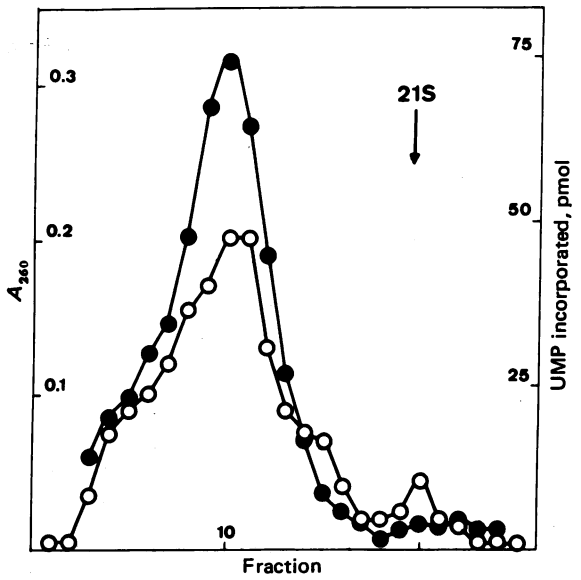

FIG. 1. Sucrose gradient sedimentation of dissociated BKV. A 50-μl aliquot of viral suspension (containing 15 μg of DNA) was treated at pH 9.85 and sedimented through a 5–20% sucrose gradient containing 0.1 M NaCl, 10 mM Tris·HCl (pH 7.5), 1 mM EDTA, and 0.25% Triton X-100. Aliquots (50 μl) of each fraction were used for absorbance determination (○) or added to a transcription reaction mixture with *E. coli* RNA polymerase to measure template activity (●). Sedimentation is from right to left.

fractions of the peak having the highest activity/A_{260} ratio. No endogenous RNA synthetic activity was detected in the absence of added RNA polymerase (data not shown). This experiment shows that DNA in the 55–60S nucleoprotein complex is available for transcription.

We then compared the template efficiency of BKV minichromosome to that of purified superhelical viral DNA. Fractions with the highest template activity (fractions 9–11) were pooled and transcribed in parallel with an equivalent amount of deproteinized BKV DNA. Incorporation kinetics (Fig. 3) showed that, after 30 min, minichromosome-directed RNA synthesis amounted to 20% of that with purified DNA. Thus,

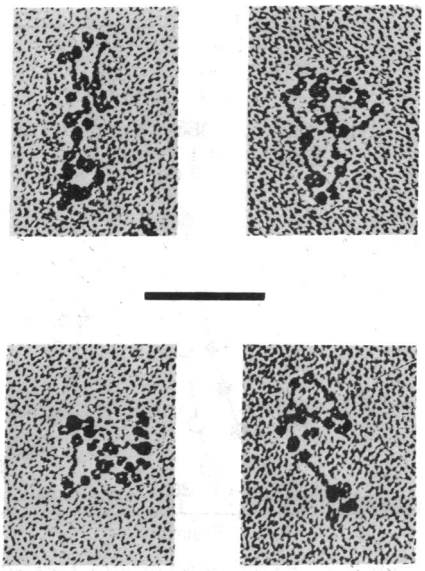

FIG. 2. Electron micrographs of BKV nucleoprotein complexes extracted from purified virions. Sample is from fraction 10 of sucrose gradient shown in Fig. 1. Bar represents 200 nm.

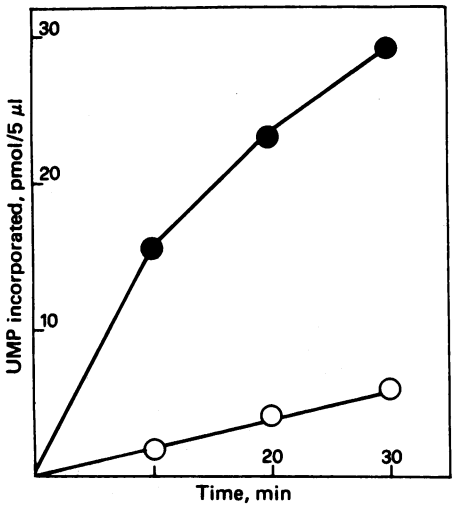

FIG. 3. Transcription kinetics of BKV minichromosome as compared to superhelical BKV DNA. Aliquots (50 μl each) of fractions 9, 10, and 11 from the sucrose gradient in Fig. 1 were pooled and added to a transcription reaction mixture. An equivalent amount of purified viral DNA (1.4 μg) was added to a parallel reaction. Incorporation of [^{3}H]UMP (specific activity 5×10^5 cpm/nmol) was determined on 5-μl samples at the indicated times. ○, Complex-directed synthesis; ●, purified DNA-directed synthesis.

the presence of nucleosomes on the viral DNA results in a strong, though not complete, inhibition of transcription. The possibility of RNA degradation by contaminating RNase activity could be eliminated by appropriate controls (unpublished observations).

How do nucleosomes interfere with transcription? In order to see whether RNA chain elongation was inhibited on the minichromosome, we compared the size of RNA made on the complex with that made on DNA. Sedimentation under denaturing conditions (Fig. 4) showed that the two RNAs were of different size: the one made on purified DNA had an average sedimentation coefficient of 16 S, as compared to 13 S for RNA made on the complex. This indicates that RNA chain elongation is inhibited by the presence of nucleosomes on DNA. However,

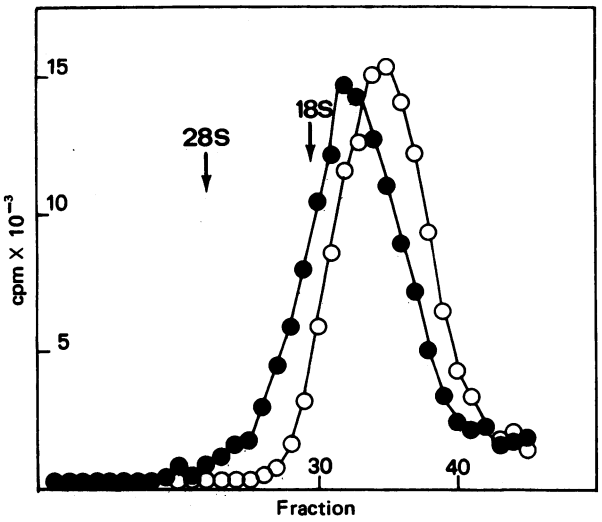

FIG. 4. Size of RNA synthesized *in vitro*. Purified RNAs made on nucleoprotein complex (○) or on purified BKV DNA (●) after 30 min of synthesis were purified, denatured in formamide, and sedimented through formamide/sucrose gradients.

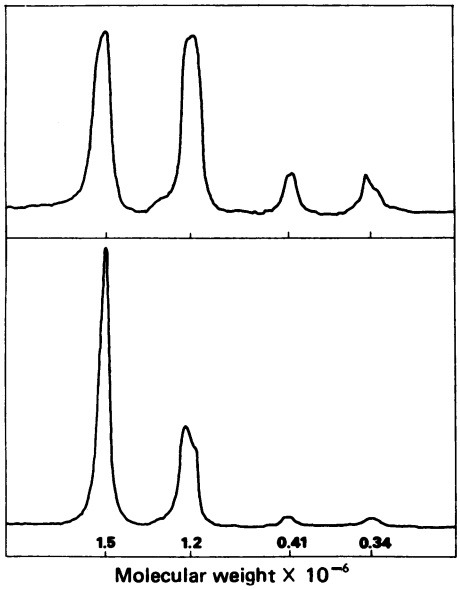

FIG. 5. Hybridization of RNA synthesized *in vitro* to separated *Hin*dIII fragments of BKV DNA. ^{32}P-Labeled RNA made *in vitro* after a 30-min synthesis on BKV minichromosome or superhelical DNA was purified and treated with DNase. [^{32}P]UTP had a specific radioactivity of 3.8×10^5 cpm/nmol. RNA was then added ($3–4 \times 10^5$ cpm) to 2 ml of 0.3 M NaCl/30 mM Na citrate in a glass scintillation vial. A 5-mm-wide nitrocellulose strip on which denatured *Hin*dIII fragments of BKV DNA had been transferred (26) was then added to the vial, which was sealed and incubated at 70° for 24 hr. The strip was then washed extensively in 0.9 M NaCl/90 mM Na citrate, treated with RNase, washed again in 0.3 M NaCl/30 mM Na citrate, dried, and laid on Kodak Industrex Film for radioautography. After a 24-hr exposure the film was developed and scanned with a densitometer. Before transfer from agarose to nitrocellulose, bands containing fragments were cut and set apart in order to obtain cleaner hybridization patterns. Relative positions of bands are therefore not representative of electrophoretic mobility. (*Upper*) RNA made on purified DNA; (*Lower*) RNA made on nucleoprotein complex.

the relatively small difference in chain length does not quantitatively account for the 5-fold difference in total RNA synthesis (Fig. 3). One must therefore conclude that the decreased template activity of the minichromosome relative to purified DNA is due to inhibition of both initiation and elongation of RNA chains.

Hybridization analysis of RNA

Does the presence of nucleosomes allow selective transcription of defined DNA sequences? BKV DNA is cut by restriction endonuclease *Hin*dIII into four fragments, with molecular weights 1.5, 1.2, 0.41, and 0.34×10^6 (5). When ^{32}P-labeled RNA made on purified viral DNA was hybridized to the separated fragments by the Southern technique (26) the amount of hybridization to each fragment was roughly proportional to the fragment's size (Fig. 5 *upper*). This indicates that with superhelical DNA as the template, all regions of BKV genome are equally transcribed. When BKV minichromosome was the transcription template (Fig. 5 *lower*), hybridization to all four fragments was again detected. In this case, however, relative amounts of RNA hybridized were not as expected from the size of each fragment. Transcription appeared to occur preferentially on the region corresponding to the largest fragment, whereas that corresponding to the two smallest ones was very poorly transcribed. Thus, although the presence of nucleosomes along the DNA does not confer an absolute sequence specificity to transcription, it affects differently different regions of the

genome. Possible interpretations and implications of this finding will be discussed in the next section.

In order to see whether strand selection of *in vitro* transcription was increased by the presence of nucleosomes, we compared the self-complementarity of RNAs made on naked DNA and on the complex. After exhaustive self-hybridization, both transcripts contained approximately 60% of input RNA that had become RNase resistant (not shown), indicating that no selective transcription of one or the other DNA strand was occurring on the minichromosome.

DISCUSSION

We have shown that BKV DNA is present inside the virions as a nucleoprotein complex consisting of about 20 nucleosome units aligned on the DNA molecule. This complex is similar to that recently isolated from SV40 particles (18) and to viral minichromosomes found in cells infected with SV40 and polyoma virus (15–17).

Association with histones in nucleosomes appears to be a constant feature of papovavirus DNA throughout the viral cycle. In fact, practically all viral DNA is found in such complexes late in infection, when active viral replication and transcription are occurring (16, 19). Protein/DNA ratio in the complex has been reported not to vary during DNA replication (16, 27, 28). It was of interest, therefore, to investigate the properties of BKV minichromosome as a transcription template. Previous attempts to reproduce SV40 or polyoma virus transcription in *in vitro* systems with deproteinized viral DNA have failed (29–32). Transcription of a 35–40S complex isolated from SV40 particles at pH 10.5 has been studied by Huang *et al.* (21). It is now known, however, that treatment at this pH partially disrupts the nucleosome structure and yields complexes in which a large fraction of histones has been dissociated from DNA (22). Some workers were able to isolate viral transcription complexes from SV40-infected cells (19, 33). Limited elongation of preinitiated RNA chains could be carried on in such complexes by an associated RNA polymerase.

The experiments we report here show that BKV minichromosome can act as a template for *E. coli* RNA polymerase, although with a lower efficiency with respect to purified superhelical DNA. Size analysis of the RNA product shows that chain elongation is inhibited by the presence of nucleosomes along the DNA. Size reduction, however, does not account for the observed lowering of nucleotide incorporation to 1/5 its original value. In fact, the average length of RNA molecules transcribed from the complex (12–13 S, corresponding to approximately 800–950 nucleotides) is much higher than what would be expected if only histone-free, internucleosomal DNA (40–55 base pairs, see refs. 15 and 16) were transcribed. The possibility that in our experiments transcription occurred preferentially on a minority of DNA molecules with few or no nucleosomes is made unlikely by electron microscopic visualization and sedimentation properties of the isolated complex. It appears, therefore, that transcribing RNA polymerase can somehow "go past" a few nucleosomes, although with a limited efficiency. Whether this happens by nucleosome disruption, by transcription of DNA exposed on the outside of nucleosomes, or by a "push on" mechanism, by which one or more nucleosomes are displaced by advancing RNA polymerase, cannot be inferred from our results.

Our data suggest that nucleosomes also limit initiation of RNA chains, presumably by masking or structurally altering base sequences on the DNA. Another factor that should be taken into account when analyzing template activity of a minichromosome is the effect of nucleosomes on DNA conformation. Germond *et al.* (34) have shown that nucleosomes modify the winding of SV40 superhelical DNA, so that the viral minichromosome appears as a relaxed structure without superhelical turns on internucleosomal DNA (see also ref. 35). Since relaxed circular DNA molecules are less efficient transcription templates than their superhelical allomorphs (31, 32, 36), it is conceivable that the relaxed conformation of internucleosomal DNA contributes to the reduction of template activity.

Do the limitations imposed by nucleosomes give rise to a more specific transcription of the BKV genome? A regulatory role for histones in eukaryotic gene expression has been suggested for a number of years. In studies with cellular chromatin *in vitro*, histones have been regarded as transcription inhibitors, whereas nonhistone nuclear proteins have been reported to act as positive regulatory elements (for a collection of relevant papers, see ref. 37). However, whether actively transcribed cellular genes do or do not contain nucleosomes is still open to discussion. Complexity of cellular chromatin and sequence redundancy make it difficult to distinguish specific regions from the bulk of chromatin. Our finding that on BKV minichromosome some regions are better transcribed than others (Fig. 5 *lower*) indicates that the effect of nucleosomes on transcription can vary for different portions of BKV DNA. One possible explanation for this could be that nucleosome stability depends on factors such as DNA base sequence or composition. According to this interpretation, in regions of lower stability, nucleosomal DNA would be more accessible for transcription. RNA polymerase could more easily displace or bypass or even disrupt nucleosomes in these regions. Another possible interpretation of our results could be that since elongation is limited on the minichromosome, the amount of transcription for each region simply reflects the distribution of initiation sites on BKV DNA. This possibility was made rather unlikely by transcription of *Hin*dIII fragments of BKV DNA. The hybridization pattern of the RNA produced (not shown) was indistinguishable from that of Fig. 5 *upper*, suggesting a rather homogeneous distribution of initiation sites on viral DNA. Any speculation about possible specific effects of nucleosomes on transcription, however, has to deal with the fact that these repeating units are apparently randomly located on the minichromosome (38). If this is so, it is hard to imagine, for example, that nucleosomes block specific initiation sites on DNA. Preferential transcription of a region of the minichromosome is more likely a property of the entire region rather than the result of a site-specific event.

In conclusion, the experiments presented here show that minichromosomes from papovaviruses can be used as templates in transcription studies. Their small size should make them useful model systems for the study of the role of histones and nonhistone nuclear proteins in eukaryotic transcription.

Note Added in Proof. A recent paper (39) has shown that SV40 minichromosomes are not physically altered after transcription with saturating amounts of *E. coli* RNA polymerase. This strongly suggests that the *in vitro* transcription process does not cause nucleosome disruption.

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