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Nuclease Bal-31 mapping of proteins bound to a tRNA^{tyr} gene in SV40 minichromosomes

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ABSTRACT

We have analyzed proteins bound to active and to inactive tRNA^{tyr} genes imbedded in the late coding region of SV40 minichromosomal DNA. Bal-31 nuclease resection from the 5′ and 3′ sides of the active tRNA^{tyr} gene reveals proteins bound to the 5′ flank, to the promoter 'A' block, to an intragenic sequence, to the promoter 'B' block and to a 3′ downstream terminator/pause sequence. The proteins bound near the promoter 'B' block and the downstream terminator/pause sequence are reduced or eliminated by an inactivating deletion in the tRNA^{tyr} 'B block'. That proteins are detected in the 5′ flank and over the promoter 'A block' of the inactive gene contrasts with current notions regarding the requirement for a functional 'B' block for binding of transcription factors.

INTRODUCTION

Multiple factors are required for the assembly of transcription complexes on animal cell tRNA genes. The proteins defined by *in vitro* transcription assays minimally include RNA polymerase III (PolIII), TFIIIC (1&2) and TFIIIB. (For recent reviews of these studies, see Ref 1&2) A requirement for upstream sequence elements for transcription of many tRNA genes suggests that factors beyond those which associate to the intragenic control elements help determine transcription activity. Additionally, the mammalian La protein strongly influences transcription *in vitro* (3); and recent evidence supports the involvement of an RNA component for transcription (4).

Superimposed upon the function of gene-specific factors in transcription of tRNA genes is the effect of chromatin structure. Remarkably, histones may themselves be components of PolIII transcription complexes (5). Nucleosomes help determine the accessibility of gene promoter elements, and may affect transcription elongation (6). With transcription by RNA Polymerase II, DNA assembly into chromatin accentuates the importance of transcriptional activators (7) and the same may be true for transcription by PolIII.

Studying how tRNA transcription is regulated *in vivo* is problematic because of these numerous and varied effectors. To complement the limitations of *in vitro* transcription assays, we have attempted to identify the proteins utilized to transcribe a tRNA gene as it is found in the cell nucleus. A precedent for our approach is provided by studies of the yeast tRNA sup53 gene (8,9). Although in animal cells, the lack of good genetics, the size of the genome and the complexity of chromatin combine to make study of such problems more difficult, we have alleviated some of these constraints through the use of a tRNA^{tyr} gene imbedded in the late region of the SV40 genome (10). The basis for this approach derives from studies of the SV40 genome and its use as a vector, both of which have been the focus of the Berg laboratory for numerous years.

MATERIALS AND METHODS

DNAs

pSVtT-2 is a pBR322-SV40 chimera with a 263 bp *X. laevis* DNA containing a tRNA^{tyr} gene (10). The *X. laevis* sequences replace SV40 late DNA from nt 343 (HhaI) to nt 832 (HaeII); pSVtT-2 DNA also lacks SV40 late sequences from nt 1046 to nt 1782 (Fig. 1A). The tRNA^{tyr} 'B' promoter element was deleted in pSVtT-2Δ by resection of pSVtT-2 DNA from the unique MstII site within the tRNA^{tyr} gene using T4 DNA polymerase and exonuclease VII in the presence of dTTP. This generated a 13 bp deletion from nt 44 to nt 58 of the tRNA^{tyr} gene (Fig. 1B and 1C); the structure of this construct was determined by DNA sequencing.

Either of two SV40 DNAs were used to complement the pSVtT-2 and pSVtT-2Δ DNAs for the production of virus stocks. pSV-rINS-7 has an intact late region and an insertion/deletion in the early region (10). TsC219 has an intact early region but encodes a thermolabile VP1 protein (11,12). To render defective the early region, an eight base pair insertion was introduced at the unique SV40 BclI site by Klenow fill-in and ligation. The desired DNA (tsC219/Bcl^{in}) was identified by its resistance to BclI and lack of infectivity.

Preparation of minichromosomes

BSC-1 or BSC-40 cells grown in Eagle's minimal essential medium (MEM) (supplemented with essential and nonessential amino acids and vitamins, 10 mM Hepes, pH 7.2, antibiotics and 10% fetal calf serum) were infected with SVtT chimera and helper virus just at confluency. In some experiments, BSC-1 cells were used with virus stocks containing the SV-rINS-7 helper; they were incubated at 37°C and minichromosomes were isolated at 24–48 hrs post-infection. Usually, BSC-40 cells were infected

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with virus stocks containing the tsC219/Bcl^{in} helper; they were incubated at 39.5°C and minichromosomes were isolated at 44−48 hrs post-infection. The latter infections produced larger amounts of minichromosomes without accompanying virus maturation (11).

At the time of harvest, all procedures were performed at 4°C except where noted. Plates of infected cells were placed on ice; the cells were washed with ice-cold TD buffer (25 mM Tris-HCl pH 7.4, 136 mM NaCl, 5 mM KCl, 0.1% dextrose and 0.7 mM Na_2HPO_4) scraped into a small volume of TD, centrifuged at 800×g for 5 min and thoroughly resuspended at 10^7 cells/ml in TD. One-tenth volume of 5% NP-40 was added, the cells were vortexed briefly and placed on ice for 10−13 min (13). After cell lysis the nuclei were pelleted (1000×g for 5 min) and unless otherwise noted, resuspended in hypotonic buffer (TEG) containing 10 mM Tris-HCl pH 7.9, 0.5 mM EDTA, 12.5% glycerol at 1.5×10^7 nuclei/100 µl. The nuclei were immediately transferred to a microfuge tube using a yellow pipette tip and soaked for 20−60 min on ice. They were pelleted for 30 seconds at 900×g and the supernatant (containing minichromosomes) was transferred to a fresh tube and used immediately.

Generally, 1.5×10^7 nuclei yielded 3 µg (from BSC-1 cells) to 12 µg (from BSC-40 cells) of SV40 DNA as minichromosomes in a volume of 100 µl. This represents approximately 80% of the viral DNA within the cells, of which 50% is SVtT DNA (the remainder being helper DNA). These cells and their nuclei vary considerably in their sensitivity to virus infection and to lysis by NP-40. After extensively passaging the cells, the yield of minichromosomes is markedly reduced.

Transcription Assays

Transcription by minichromosomes was measured by incubating the extract from 1.5×10^7 nuclei in a solution containing 12 mM Tris-HCl pH 7.9, 150 mM KCl, divalent cations as specified, 0.5 mM EDTA, 2.5 mM dithiothreitol (DTT), 0.4 mM each ATP, UTP and CTP, .025 mM [α-^{32}P]GTP (410 Ci/mMole), 0.5 µg/ml α-amanitin and 12.5% glycerol at 25°C, unless noted otherwise. Generally, reactions were terminated after 60 min by adding SDS to 0.5%, EDTA to 10 mM and sodium acetate pH 5.1 to 50 mM. Carrier tRNA was added, the solution deproteinized and the nucleic acids precipitated with ethanol.

S-100 extracts were prepared from human 293 suspension cells as described by Yoshinaga et al (14) with slight modifications. Cells were washed once with phosphate buffered saline (PBSa) pH 7.2 containing 136 mM NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 and once with isotonic buffer containing 10 mM Hepes pH 8, 96 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT prior to lysis. Extracts (containing approximately 7 mg/ml protein) were stored in 32 mM Hepes pH 8.0, 178 mM KCl, 4.4 mM $MgCl_2$, 4.4 mM DTT and 20% glycerol. When S-100 nuclear extracts were added to the minichromosomes, the transcription mixture contained 18 mM Hepes pH 8.0, 72 mM KCl, 0.7 mM $MgCl_2$, 2 mM DTT, 0.5 mM each of ATP, CTP, GTP, 0.025 mM [α^{32}P]UTP (2 Ci/mMole), 1 µg/ml α-amanitin and 9% glycerol, and the incubation was performed for 90 min at 30°C.

Analysis of transcription products

Products of transcription assays were treated with proteinase K and extracted with phenol before fractionation on 10% polyacrylamide gels (38:2 acrylamide to bis-acrylamide) containing 8 M urea and TBE buffer (80 mM Tris- borate, 10 mM EDTA, pH 8.3). Alternatively, transcription products were hybridized to M13 single stranded DNAs containing a 410 bp (HaeII) *X. laevis* DNA fragment encoding the tRNA^{tyr} gene (15) fixed to nitrocellulose filters, essentially as described previously (16).

Restriction Endonuclease Cleavage and Bal-31 nuclease resection of minichromosomes

Minichromosomes were isolated at 44 hrs post-infection in buffer containing 0.1 mM PMSF, 10 mM Tris-HCl pH 7.9, 0.5 mM EDTA and 12.5% glycerol. They were incubated at 37°C for 5 min in buffer containing KCl (as specified) in the presence of a two to three fold excess of supercoiled pBR322 DNA to exchange nucleosomes (17) and then desalted over 1 ml Biogel P-10 (Biorad Laboratories) columns containing 10 mM Tris-HCl pH 7.9, 2 mM DTT and 0.1 mM PMSF. The DNAs were made 10 mM in $MgCl_2$ and 100 µg/ml bovine serum albumin and incubated with excess restriction enzyme (KpnI or HaeII) for 60 min at 37°C. NaCl was then added to 250 mM, $CaCl_2$ to 12 mM, and nuclease Bal-31 to 5−6 u/ml. Following incubation for 60 min at 30°C, the Bal-31 nuclease resection was stopped by adding SDS to 1% and EDTA to 25 mM. The DNA was deproteinized, ethanol precipitated and then digested with a second restriction endonuclease (either HaeII or HinPI) to generate a terminus for indirect end-labeling (18,19). The DNAs were deproteinized, treated with RNAseA and then electrophoresed through a 6% polyacrylamide gel in TBE for sufficient time to allow the xylene cyanol dye to move 4.5−5.5 cm. The gel was boiled for 15 min in TBE in a plastic pouch and then submerged in ice water. The DNAs were

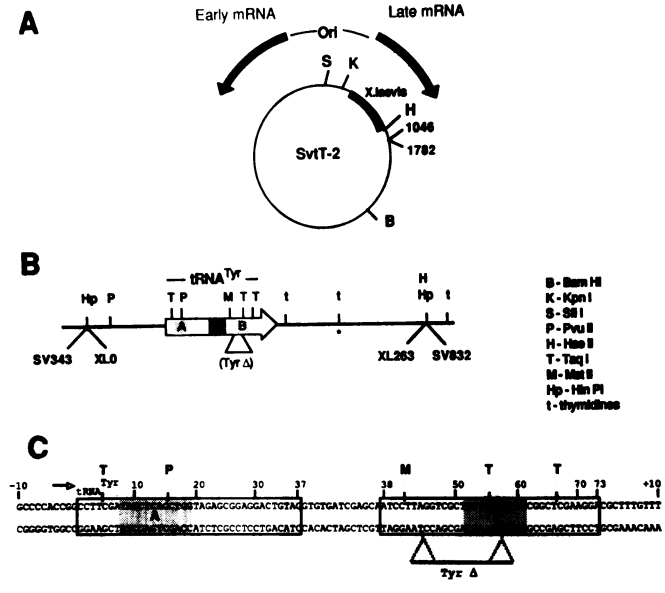

Fig. 1. Physical Maps of SVtT-DNA. A) Map of the SVtT-2 genome indicating the location of *X. laevis* DNA sequences relative to viral early and late transcription units. B) Schematic of the *X. laevis* DNA insert. Arrow indicates location and direction of transcription of tRNA^{tyr} Stippled areas indicate the approximate location of tRNA^{tyr} gene promoter 'A and B Boxes'. The deletion introduced into the promoter 'B box' of pSVtT-2 DNA is underlined. Numbers below and at either side indicate nucleotides of SV40 DNA deleted ($SV_{343}-SV_{832}$) and nucleotides of inserted *X. laevis* DNA (XL_0-XL_{263}). C) Sequence of the tRNA^{tyr} gene,indicating direction of transcription (small arrow), location of exons (boxed sequences), and extent of deletion in SVtT-2Δ DNA.

electrophoretically transferred to nylon membranes (Biotrans, ICN) at 1.2 A in 1×TBE for 35 min. The membranes were hybridized at 60°C for 18–24 hrs with radiolabeled probes prepared by extending DNA primers (15–23 nts) complementary to sequences abutting the second restriction enzyme cleavage site.

To detect the helper SV40 DNAs present within the minichromosome preparations, the filters were stripped by washing in 50% fromamide at 65°C and rehybridized with probes specific for SV40 sequences deleted from the SVtT-2 genome (nt 343 to 832).

Measurement of Restriction Endonuclease Susceptibility of SVtT-2 Minichromosomes

Minichromosomes were extracted as described above, treated with KCl at 37°C in the presence of excess pBR322 DNA and then desalted over Biogel P10 columns. Following digestion with excess restriction enzyme (BamHI, SfiI, HaeII or HinPI) for 60 min at 37°C, the nucleic acids were phenol extracted and precipitated. The DNAs were fractionated through agarose gels and hybridized with probes complementary to the *X. laevis* insert. The proportion of each form of DNA following digestion was estimated from the autoradiograph band intensity.

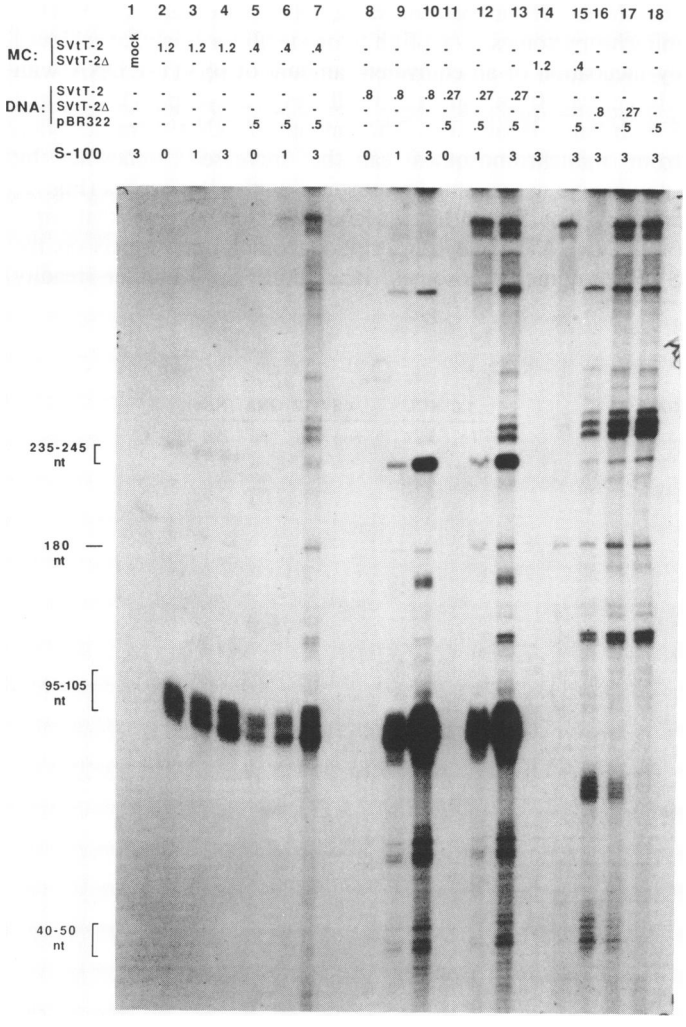

Fig. 2. Transcription Analysis of SVtT Minichromosomes and DNAs. Minichromosomes (MC) prepared from cells infected with SVtT-2 or SVtT-2 viruses or from mock infected cells were incubated in transcription buffer containing 0.7 mM $MgCl_2$ without or with added pBR322 DNA and without or with 1 or 3 μl S100 extract. DNAs (in μg of pSVtT-2 or pSVtT-2Δ or pBR322 DNA) were incubated with transcription buffer without or with S100 extract. Transcripts were analyzed by gel electrophoresis.

RESULTS

The *X. laevis* $tRNA^{tyr}$ gene inserted in the late region of SV40 (Fig. 1; SVtT-2DNA) is abundantly expressed within infected monkey cells (10). Lassar et al (20) showed that a similar SV40 construct containing a $tRNA^{met}$ gene formed minichromosomes containing stable transcription complexes including TFIIIC and TFIIIB, but lacking PolIII. With these observations in mind, we asked whether SVtT-2 minichromosomes containing the $tRNA^{tyr}$ gene could be used to map the proteins associated with an actively transcribed gene in mammalian cells.

SV40 SVtT-2 minichromsomes contain functional $tRNA^{tyr}$ transcription complexes

To reduce the number of mature and partially encapsidated virions included in minichromosome preparations, we isolated nuclei from infected cells before viral late proteins had time to accumulate, or we prevented viral late protein synthesis through the use of a helper virus (tsC219/Bcl^{in}) which encodes an inactive VP1 at 39.5°C. Intracellular SV40 nucleoprotein complexes were isolated by soaking the nuclei of infected cells in hypotonic buffer. When these nuclear extracts were incubated

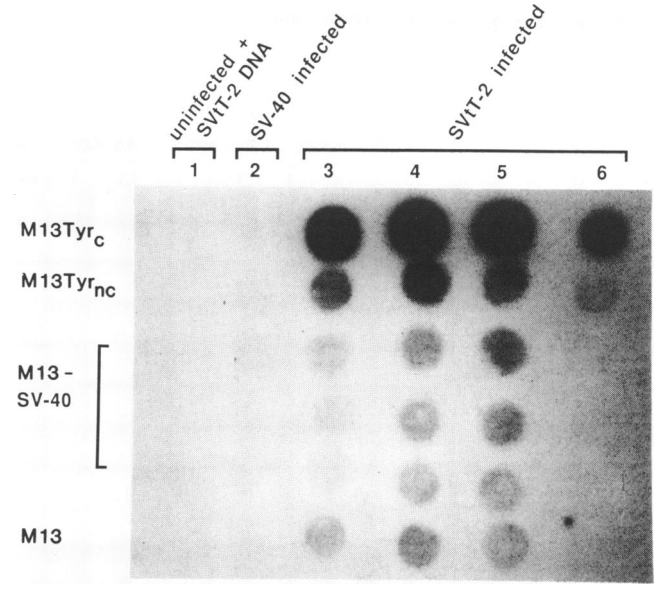

Fig. 3. Hybridization Analysis of Transcription Products of SVtT-2 Minichromosomes. RNAs synthesized by SVtT-2 or SV40 minichromosomes or by 'mock' nuclear extracts incubated in transcription buffer containing 5 mM $MgCl_2$ and 2 mM $MnCl_2$ were hybridized to single stranded M13 DNAs containing a *X. laevis* HaeII fragment with $tRNA^{tyr}$ coding (M13tyr$_c$) or noncoding (M13tyr$_{nc}$) sequences, or containing SV40 late DNA coding sequences (nt 1046–3496 on separate fragments). Column 1: Nuclear extracts isolated from uninfected cells were incubated with pSVtT-2 DNA for 60 min. Column 2: Minichromosomes from SV40 infected cells were incubated for 60 min. Columns 3–6: Minichromosomes isolated from SVtT-2 infected cells were incubated in transcription buffer containing ribonucleoside triphosphates for 30, 60 and 120 min, respectively, (columns 3, 4, 5) or with transcription buffer lacking ribonucleoside triphosphates for 30 min before their addition for 30 min (column 6).

with ribonucleoside triphosphates in the presence of α-amanitin, $tRNA^{tyr}$ transcripts of 95–105 nt were actively synthesized (Fig. 2, lanes 2–7); similar products were observed upon transcription of purified pSVtT-2 DNA template with a human 293 cell S100 extract containing PolIII (Fig. 2, lanes 8–13). These transcripts have previously been characterized as containing the mature $tRNA^{tyr}$ with a 13nt intron and 3′ and 5′ flanking sequences (21). We also observed larger transcripts of 235–245 nts with both types of templates, and found they reflect transcription through the t-rich sequence proximal to the 3′ end of the tRNA gene (data not shown). Transcripts containing $tRNA^{tyr}$ were not observed upon incubation of nuclear extracts from 'mock' infected cells with pSVtT-2 DNA (Fig. 2, lane 1), nor upon incubation of pBR322 DNA with the S100 extract (Fig. 2, lane 18). As neither the mutant SVtT-2Δ minichromosomes nor the mutant pSVtT-2Δ DNA are active as templates (Fig. 2, lanes 14–17), the $tRNA^{tyr}$ gene imbedded in SV40 DNA appears to rely upon the promoter 'B block' and concomitantly, TFIIIC (1&2) for transcription complex formation.

Table 1. Susceptibility of SVtT-2 Minichromosomes to Restriction Enzymes

Restriction Enzyme	[KCl] 0	0.6	0.7	0.8	0.9
SfiI (0)[a]	80%[b]	80%	80%	80%	80–100%
HinPI (343)	50%	60%	60%	80%	90–100%
HaeII (832)	10%	20%	20%	30%	80–90%
BamHI(2533)	40–60%	80–100%	80–100%	80–100%	80–100%

[a]Position on the SV40 physical map.
[b]Percent of total DNA cleaved to linear form.

We asked whether transcription from these minichromosomes exhibits the correct template specificity by hybridizing the newly synthesized RNAs to single-stranded *X. laevis* DNA bound to filters (Fig. 3). The labeled RNAs hybridized almost exclusively to the $tRNA^{tyr}$ coding strand (Fig. 3, columns 3–6; compare $M13tyr_c$ with $M13tyr_{nc}$). Failure of these transcripts to hybridize to M13 SV40 DNAs (or to M13 DNA) indicate they derive from the *X. laevis* insert in the $M13tyr_c$ DNA. Very importantly, we observe no transcription by minichromosomes prepared from cells infected with wild-type SV40 virus (Fig. 3, column 2) nor by similar nuclear extracts from mock infected cells which subsequently have been incubated with pSVtT-2 DNA (Fig. 3, column 1).

These data indicate that the transcription complexes present in these minichromosomes together with diffusible factors present in the nuclear supernatant are fully competent for transcribing $tRNA^{tyr}$ molecules. However, only a small fraction of the SvtT-2 minichromosomes may contain active transcription complexes. The amount of transcription observed from SVtT-2 minichromosomes is one fifth to one tenth (or less) that achieved by incubation of an equivalent amount of pSVtT-2 DNA with S100 extract from human 293 cells (Fig. 2; compare lanes 2 and 5 with lanes 10 and 13). The amount of $tRNA^{tyr}$ transcription from minichromosomes can be increased somewhat by supplementing the minichromosomes with S100 extract (Fig. 2, compare lane 5 with lanes 6 and 7) which suggests that some of the $tRNA^{tyr}$ gene transcription complexes formed on the SVtT-2 minichromosomes lack PolIII or another readily

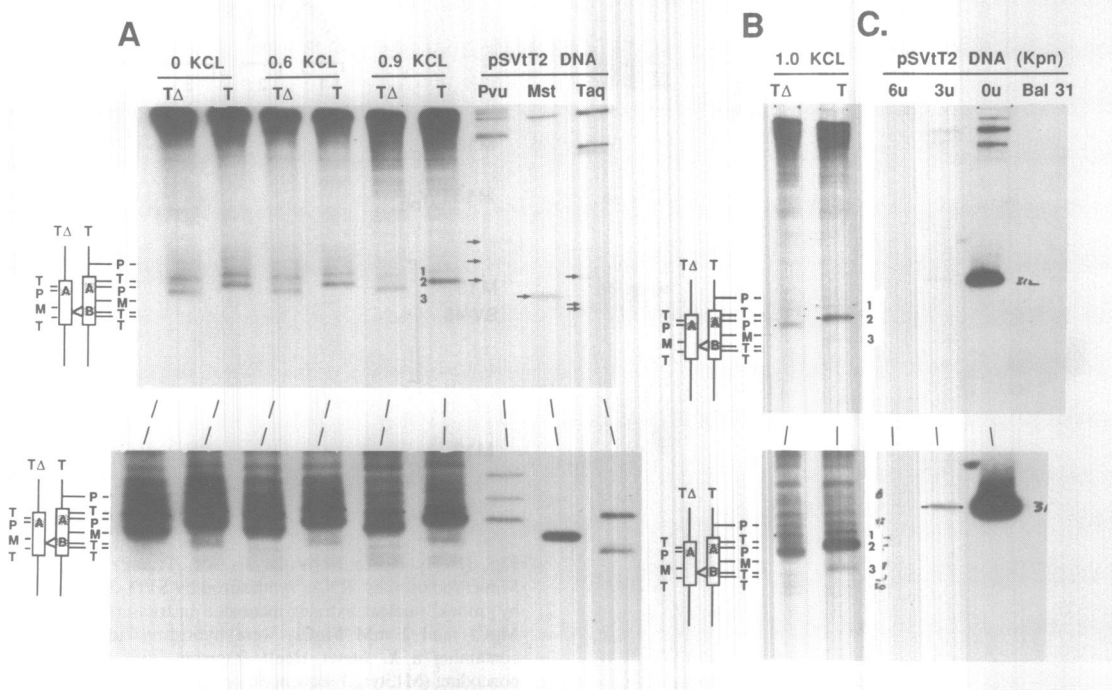

Fig. 4. Analysis of Bal 31 Nuclease Resection from the 5′ Side of the $tRNA^{tyr}$ Gene. DNA fragments resulting from Bal-31 nuclease digestion from the KpnI site and HaeII digestion were transferred to Biotrans membranes and hybridized with a probe abutting the HaeII site. Panel A: SVtT-2Δ (indicated by TΔ) or SVtT-2 (indicated by T) minichromosomes washed with buffer containing O M KCL, 0.6 M KCL or 0.9 M KCL. pSVtT-2 DNA partially digested with PvuII, MstII or TaqI and completely digested with HaeII were fractionated on the same gel to provide markers for sites within the *X. laevis* DNA, shown by the schematic to the left of the autoradiograms (see Fig. 1B, C for greater detail). Lower panel provides a darker exposure of the same gel lanes. Panel B: Minichromosomes washed with 1.0 M KCL electrophoresed in a separate gel from that shown in Panel A. Panel C: pSVtT-2 DNA digested with KpnI and treated with 0 units, 3 units or 6 units of Bal-31 nuclease prior to digestion with HaeII and electrophoresis.

dissociable factors, or that the extract in combination with nonspecific pBR322 DNA removes inhibitory substances.

Stability of proteins bound to SV40 minichromosomes

Transcription complexes remain bound to tDNAs and 5S DNAs in solutions of high ionic strength (22–26). The stability of such complexes exceeds that of nucleosomes, which will exchange to supercoiled naked DNA at 0.8 M cation (17). We reasoned that this property might be exploited to strip SVtT-2 minichromosomes of nucleosomes and loosely bound non-histone proteins, permitting the identification of $tRNA^{tyr}$ transcription complexes through their resistance to nucleases.

To follow the dissociation of histones and other proteins from the minichromosomes, we measured the susceptibility of minichromosomal DNA to restriction endonuclease cleavage. HaeII recognizes one site in SV40 coding sequences at the junction between the *X. laevis* insert and SV40 DNA (Fig. 1A). Minichromosomal DNA became susceptible to HaeII after exposure to 0.9 M KCl, indicating this is sufficient to strip nucleosomes (Table 1). (The HaeII site in SV40 DNA is a preferred nucleosome location (27)). BamHI also cleaves in SV40 coding sequences, and digested most of the minichromosomal DNA after exposure to 0.6 M KCl. SfiI and HinPI recognize sites in or adjacent to the SV40 regulatory region, and cleaved a large fraction of minichromosomes at low ionic strength. This region is largely free of nucleosomes, particularly in viral chromatin lacking SV40 late proteins (27,28).

Barriers to nuclease Bal-31 digestion through the $tRNA^{tyr}$ gene in SVtT-2 minichromosomes

Previous studies by Scott et al (29) have established that Bal-31 nuclease can be used to map proteins binding to the SV40 DNA regulatory region. We reasoned that this approach should be able to detect a transcription complex assembled on the $tRNA^{tyr}$ gene, particularly as histones and loosely bound proteins were removed by incubation of minichromosomes in buffers of increasing ionic strength. Accordingly, minichromosomes from cells infected with either SVtT-2 or SVtT-2Δ virus preparations (each containing the $tsC219/Bcl^{in}$ helper) were incubated together with pBR322 DNA in buffers of increasing ionic strength

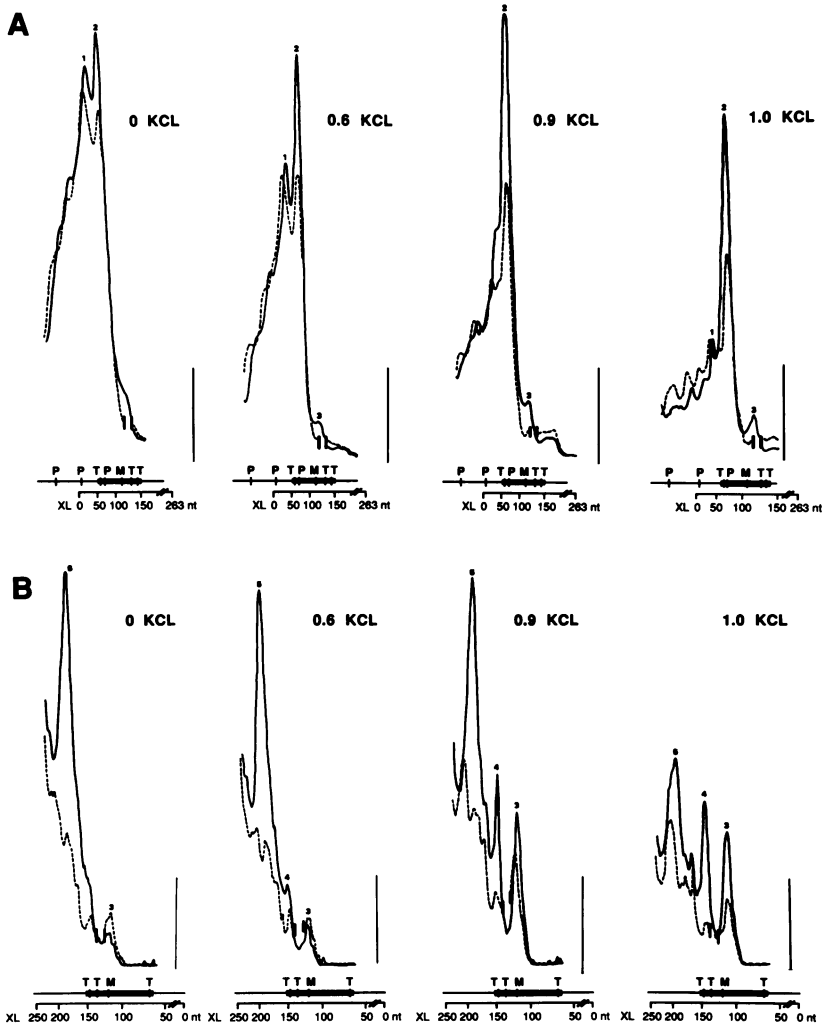

Fig. 5. Densitometric Scans of Bal-31 Nuclease Resections. Each panel displays a gel lane from Fig. 4 and Fig. 6 containing SVtT-2Δ (dotted line) or SVtT-2 (solid line) DNAs, scanned with a densitometer. The position of the SVtT-2Δ DNA has been corrected for the 13 bp deletion in the $tRNA^{tyr}$ promoter 'B block' (which is shown by the vertical bars in the tracing). Below each tracing is a line diagram of the *X. laevis* DNA sequence with the $tRNA^{tyr}$ sequence and selected restriction enzyme cleavage sites marked.

to cause exchange of nucleosomes, then desalted and digested consecutively with a restriction enzyme and Bal-31 nuclease. KpnI was used to open the SVtT DNAs upstream of the tRNA^{tyr} gene; HaeII was used to open the SVtT DNAs downstream of the tRNA^{tyr} gene (Fig. 1). Following Bal-31 nuclease resection, the DNAs were deproteinized and digested with a second restriction endonuclease whose cleavage site lies on the other side of the tRNA^{tyr} gene (HaeII or HinPI, respectively; Fig. 1). The DNA fragments (resected with Bal-31 nuclease at one end, and with the other end determined by the second restriction enzyme cleavage) were fractionated through polyacrylamide gels, transferred to nylon membranes and indirect end- labeled by hybridization to radiolabeled probes complementary to sequences abutting the second restriction enzyme cleavage site. Restriction endonuclease digested DNAs included in the same gel provided precise mobility markers on for the locations of sequences within the *X. laevis* DNA.

A representative analysis of the sites upstream of the tRNA^{tyr} gene at which Bal-31 nuclease movement is blocked by proteins is provided in Fig. 4. (Similar analyses were performed five independent times.) With SVtT-2 minichromosomes two prominent Bal-31 nuclease barriers are detected, one immediately preceding the 5′ end of the tRNA^{tyr} gene and the other within the gene, near sequences comprising the tRNA^{tyr} gene 'A' promoter element (Fig. 4, panels A and B; bands marked 1 and 2, respectively). Neither barrier is observed with minichromosomes which have not been digested with Bal-31 nuclease (data not shown). A similar doublet of Bal-31 nuclease barriers is observed at the 5′ side of the inactive tRNA^{tyr} gene in SVtT-2Δ minichromosomes, although their positions relative to the tRNA^{tyr} DNA is shifted because of the deletion in the B block, (Fig. 4, panels A and B). A weak Bal-31 nuclease barrier within the gene, between the promoter 'A and B blocks' is detected primarily in the SVtT-2 minichromosomes (Fig. 4, panels A and B, band 3).

To better quantitate the relative intensities of these bands and to more accurately map their positions on the tRNA^{tyr} gene, we scanned each autoradiograph by densitometry. Fig. 5A provides such an analysis of the autoradiograms portrayed in Fig. 4A and B. It is apparent that in buffers with low salt both the SVtT-2 and SVtT-2Δ minichromosomes have protein(s) bound just upstream of the tRNA gene (band 1 in Fig. 4A and B, and in Fig. 5A) which are displaced from both the SVtT-2 and SVtT-2Δ minichromosomes by buffers of higher ionic strength. Both types of minichromosomes also exhibit a protein (band 2) bound directly to the tRNA^{tyr} gene promoter 'A block' sequences. After washes containing 1.0 M KCL this protein is still prominent in SVtT-2 minichromosomes, while in SVtT-2Δ minichromosomes washed with 1.0 M KCL, this protein may be slightly reduced relative to its initial level (Fig. 4, panel B and Fig. 5, panel A). The likely identities of these proteins is discussed in the subsequent section.

A representative analysis of the barriers to Bal-31 nuclease resection from the 3′ side of the tRNA^{tyr} gene (HaeII site) is provided in Fig. 6 and in Fig. 5, panel B. At low and intermediate ionic strengths, SVtT-2 minichromosomes uniquely exhibit a prominent barrier (band 5) downstream of the tRNA^{tyr} gene, very near a tRNA^{tyr} gene transcription termination or processing site (indicated by '*' in Fig. 1B; Scanlon & Folk, unpublished). After incubation of the minichromosomes in buffers of high ionic strength (0.9 M and 1.0 M) this barrier is diminished to the same background level observed in SVtT-2Δ minichromosomes. Within the tRNA^{tyr} coding sequences of SVtT-2 minichromosomes, two barriers are equally prominent. One of these barriers (band 3) is located in the intron between the tRNA^{tyr} gene promoter 'A and B block' (Fig. 1C); the other barrier (band 4) occurs within the tRNA^{tyr} gene promoter 'B block'. In SVtT-2△ minichromosomes, only the first of these barriers (that within the intron) is prominent. Neither of these barriers is present in minichromosomes not treated with Bal-31 nuclease (Fig. 6, panel B), nor are they detected in pSVtT-2 DNA (data not shown). Furthermore, none of the barriers described (#1 through #5) are present in SV40 helper virus minichromosomes containing an intact SV40 late region rather than the *X. laevis* tRNA^{tyr} insert, which are present with the SVtT minichromosomes (data not shown). Their Bal-31 nuclease barriers were measured by hybridizing filters with a radiolabeled probe specific for the SV40 sequences replaced by the *X. laevis* sequences in SVtT-2 DNA.

Barriers to nuclease Bal-31 digestion through the tRNA^{tyr} gene in SVtT-2 DNA

The barriers to Bal-31 digestion described above are caused by proteins bound to DNA, as they are erased with deproteinized DNA (Fig. 4C and data not shown). Addition of crude nuclear extract or S100 to naked pSVtT-2 DNA induces the formation of numerous barriers, some of which are located at the same locations as those observed in minichromosomes, but a variety of others are formed as well (data not shown). These barriers reflect specific and non-specific DNA binding proteins present in such extracts, and assessing what is significant from that which

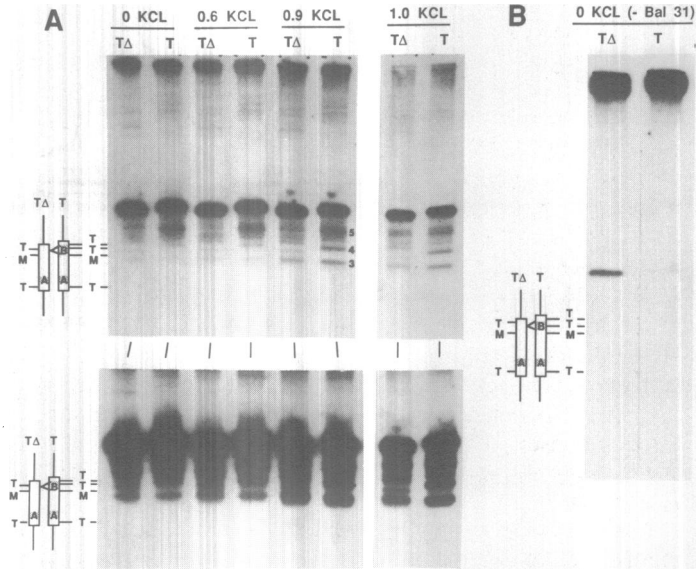

Fig 6. Analysis of Bal-31 Nuclease Resection from the 3′ Side of the tRNA^{tyr} Gene. DNA fragments resulting from Bal-31 nuclease digestion from the HaeII site and HinPI digestion were transferred to Biotrans membranes and hybridized with a probe abutting the HinPI site.Panel A: SVtT-2 (indicated by T) or SVtT-2Δ (indicated by TΔ) minichromosomes washed with buffers containing 0 M KCL, 0.6 M KCL, 0.9 M KCL or 1.0 M KCL. Schematic at the left shows the position of the tRNA^{tyr} gene determined from markers run on the same gels (not shown). The lower panel provides a darker exposure. Panel B: Minichromosomes digested with HaeII but not treated with Bal-31 were digested with HinPI and indirect end-labeled. The schematic indicates the position of the tRNA^{tyr} gene.

is not significant requires purification at the component factors and their assembly into structures analogous to that found in minichromosomes. Such an effort is underway.

DISCUSSION

The ease with which SV40 genomes can be genetically altered and their minichromosomes isolated and characterized make them particularly suitable for studying replication and transcription. Virtually all of the proteins which bind to the minichromosome prior to encapsidation are encoded by the cell. Thus, transcription complexes which assemble on the viral minichromosome should be similar if not identical to those formed on cellular DNA.

Our objective was to detect and to characterize transcription complexes formed on tRNA genes. The $tRNA^{tyr}$ gene inserted within the late- coding region of SV40 is abundantly expressed within infected cells, and viral minichromosomes faithfully express $tRNA^{tyr}$ transcripts *in vitro*. We have no reason to suspect that the transcription complexes assembled on the minichromosomes are significantly altered by the procedures used to extract them.

Within the SVtT-2 minichromosomes we detect prominent barriers to Bal-31 nuclease situated over the tRNA 'A & B block' promoter sequences known to be important for transcription complex formation *in vitro* (bands #2 and #4, respectively). The amount and stability of these proteins is altered by the introduction of an inactivating deletion in the 'B' block of the $tRNA^{tyr}$ gene, the site of binding of TFIIIC(1&2). We detected no comparable proteins on wild-type SV40 DNA (measured by probing the helper DNA in these same experiments; data not shown). This suggests that these proteins are components of the $tRNA^{tyr}$ transcription complex. Band #2 coincides with the 'A block' promoter sequences of the $tRNA^{tyr}$ gene. In the SVtT-2Δ minichromosomes, there is less of this protein, and it is more labile to high ionic strengths. It probably corresponds to the TFIIIC-1 protein which protects the tRNA gene promoter 'A block' *in vitro* (30). To the 5′ side of this barrier, an additional, less prominent Bal-31 nuclear barrier (band 1) is located on both the $tRNA^{tyr}$ and the $tRNA^{tyr}\Delta$ genes. It may comprise upstream protein(s) which help determine transcriptional competence of tRNA genes (31,32). We believe this protein is unlikely to be TFIIIB, whose association with DNA relies upon binding of TFIIIC (26,33) as this is unlikely to occur with the deleted 'B' block in SVtT-2Õ DNA. A third barrier (band #3) is located within the $tRNA^{tyr}$ gene, between promoter elements A and B. Its identity is as yet unclear. Genetic and biochemical studies of several tRNA genes suggest that this region can be important for their transcriptional competence, and recent UV-crosslinking studies suggest a component of TFIIIC may interact with this region (34).

Analysis of the barriers encountered by Bal-31 nuclease as it digests from the 3′ side of the $tRNA^{tyr}$ gene reveals a prominent barrier (band #5) coinciding with a RNA polymerase III transcription termination or processing sequence (Scanlon & Folk, unpublished). This protein occurs primarily on SVtT-2 minichromosomes, suggesting that it is associated with their transcriptional activity. We speculate that protein(s) at this site are involved in transcription termination or processing (a role suggested for the La protein; Ref #3), and might include paused RNA polymerase III molecules. Coinciding with the $tRNA^{tyr}$ gene promoter 'B block' is a prominent Bal-31 nuclease barrier (band #4) which is absent or greatly diminished in the SVtT-2Δ minichromosomes. This may correspond to the mammalian TFIIIC-2 protein(s) which bind to the tRNA 'B block' sequences *in vitro* (30,35).

If the assignments of these proteins is correct, it provides some insights and surprises. The 'B' block intragenic promoter element is the major quantitative determinant of tRNA gene promoter strength; thus, the logical reason for the transcriptional inactivity of the $tRNA^{tyr}\Delta$ gene is the failure of TFIIIC protein(s) to bind to the deleted promoter 'B block', and this is consistent with the lack of band #4 in $tRNA^{tyr\Delta}$. However, deletion of this promoter element does not appear to prevent association of proteins with the promoter 'A block' nor to the tRNA gene 5′ flanking sequences in the $tRNA^{tyr\Delta}$ minichromosomes. This is particularly noteworthy for several reasons. First, the properties of TFIIIC (1&2) purified from human cells suggest that binding to the 'B' block is prerequisite for binding to the A block (1,2,14,35). Second, the yeast TFIIIB appears to be strongly bound to upstream tRNA sequences after being positioned by TFIIIC (26) and genomic footprinting of yeast tRNA genes containing inactivating mutations in the 'B' block cause a loss of protection over 5′ flanking sequences (9). This is clearly not the case here; were this the case for this $tRNA^{tyr}$ gene, we should have observed a strong Bal-31 nuclease barrier upstream of the active gene, and loss of that barrier in the inactive gene. The failure to observe such a dramatic difference between the proteins associated with the 5′ flanking sequences of the $tRNA^{tyr}$ gene and its inactive counterpart, $tRNA^{tyr}\Delta$, suggests that mammalian TFIIIB does not form the highly stable complex with upstream sequences that has been reported for yeast genes. For yeast tRNA transcription, the stability of TFIIIB bound to DNA enables it to act as a sole transcription initiation factor, independent of TFIIIC (26). This may not be true for mammalian TFIIIB. That pol III transcription complexes might assemble by alternative pathways has previously been suggested for several animal cell systems (36,37).

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