Localization of T-antigen on simian virus 40 minichromosomes by immunoelectron microscopy

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SV40 chromatin extracted from 42 h post-infected cells by a modification of the standard Triton X-100-EDTA procedure and purified on neutral sucrose gradients was partially immunoprecipitable by a specific SV40 T-antigen (T-Ag) antiserum. Electron microscopic observations of spread minichromosomes were made after labelling by the indirect colloidal gold immunological method using monoclonal antibodies specific for the SV40 T-Ag. In 1–2% of morphologically mature minichromosomes the labelling corresponding to tightly bound T-Ag was localized within the nucleosome-free region near one of its borders. Mapping with three single-cut restriction endonucleases: *Bam*HI, *Eco*RI and *Bgl*II localized the labelling near to, or at the origin of, replication. In addition, we observed that the T-Ag specific antibodies were linked to a DNA-bound particle when the region was not masked by a large clump of antibodies. The variable size of this particle led us to suggest that it might be a complex of T-Ag with other proteins.

Key words: immunoelectron microscopy/SV40 large T-antigen/SV40 chromatin/origin of replication

Introduction

SV40 large T-antigen (T-Ag) is a multifunctional phosphoprotein synthesized both in productive and abortive infections (see review, Tooze, 1980). It recognizes and binds specifically to a region of SV40 DNA that contains the origin of replication and the early transcriptional promoter (Tjian, 1978; Shalloway *et al.*, 1980). This binding of T-Ag controls the initiation of DNA replication (Chou *et al.*, 1974; Tegtmeyer, 1972) and regulates early and late transcription (Cowan *et al.*, 1973; Reed *et al.*, 1976; Alwine *et al.*, 1977). The major DNA binding species of T-Ag is a tetramer (Myers *et al.*, 1981a) which binds *in vitro* in a sequential (Tjian, 1978, 1979) and cooperative manner (Myers *et al.*, 1981b) to three distinct but closely-spaced recognition regions: I, II and III which span the origin of replication. A certain variability exists in these T-Ag binding regions defined by three different groups (Tjian, 1978; Shalloway *et al.*, 1980; Tegtmeyer *et al.*, 1981). More recently, Tegtmeyer *et al.* (1983) reported that the regions I and II themselves contain multiple binding sites and that different degrees of binding to these regions resulted in different extents of protection from DNase digestion. Cooperative binding *in vitro* of T-Ag to SV40 chromatin was suggested by the studies of Persicolo-DiLauro *et al.* (1977). During a lytic infection T-Ag interacts with the viral genome associated with mature as well as replicating nucleoprotein complexes (Mann and Hunter, 1979; Segawa *et al.*, 1980; Stahl and Knippers, 1983). In replicating chromatin, a class

of T-Ag appeared to be specifically and tightly bound to a DNA fragment spanning the origin of replication (Reiser *et al.*, 1980). The viral nucleoprotein complexes isolated from lytic infected cells display a very characteristic morphological pattern. They have a nucleosomal structure similar to that of cellular chromatin (Germond *et al.*, 1975; Griffith, 1975; Bellard *et al.*, 1976; Cremisi *et al.*, 1976; Müller *et al.*, 1978; Moyne *et al.*, 1981). In addition, different methods of analysis including electron microscopy (Jakobovits *et al.*, 1980; Saragosti *et al.*, 1980), nuclease digestion (Scott and Wigmore, 1978; Waldeck *et al.*, 1978; Varshavsky *et al.*, 1979), carcinogen binding (Beard *et al.*, 1981) and psoralen cross-linking (Robinson and Hallick, 1982) all suggested that in 20–25% of the minichromosomes, a nucleosome-free region (gap) of 300–400 bp is present around the origin of replication. Indeed, the gap was mapped between 0.67 and 0.74 map units on the physical map; it encompasses the origin of replication, the 5' ends of early and late mRNA and a region critical for early gene expression (Benoist and Chambon, 1981; Ghosh and Lebowitz, 1981; Mathis and Chambon, 1981).

The aim of the present study was to characterize the association of T-Ag with SV40 chromatin *in situ* during the late phase of a lytic infection. To this end, we used the indirect colloidal gold immunological method combined with high resolution observations of spread minichromosomes. The present studies suggest that 1–2% of the viral chromosomes have tightly bound T-Ag preferentially associated with the naked DNA in the gap region. The T-Ag specific immunolabelling was localized near to or at the origin of replication in association with a visible DNA-bound particle.

Results

Association of large T-Ag with SV40 chromatin

Our modifications of the method of Varshavsky *et al.* (1977) allowed us to preserve partially the association of T-Ag with viral chromatin, which could be well spread for high resolution observations. Two SV40 nucleoprotein complexes could be separated on the sucrose gradient in which viral chromosomes sedimented at 70S and previrions and dissembled virions at around 120S (Garber *et al.*, 1980). Following 5 min labelling of cells with [³H]thymidine, 42 h after infection, our extraction procedure at pH 7.3 revealed that the pulse-labelled replicating chromatin sedimented in the heavier fractions of the peak containing the 70S mature minichromosomes. As observed by electron microscopy, these labelled fractions contained <2% of replicative intermediates.

Hamster SV40 T-Ag antiserum used under conditions that specifically precipitated T-Ag protein showed that ~30% of the total amount of the 90-K T-Ag was found in the nucleoprotein complex preparation, while 30% represented the T-Ag in the cytoplasmic fraction and 40% in the residual nuclear content after extraction. Partial purification on neu-

Fig. 1. T-Ag labelling on SV40 minichromosomes. (A) General view showing that only one nucleoprotein complex is labelled at a single site of the gap (arrow). No non-specific contamination of the preparation by colloidal gold particles is observed. (B–G) A single labelled site is revealed on these minichromosomes: it is localized near one limit of the gap except in E. In some cases (G) one additional unlabelled DNA-bound particle is also observed (arrow). Tungsten rotary shadowing; bar = 100 nm.

tral sucrose gradients of nucleoprotein complex preparations revealed that ~10% of their 90-K T-Ag content co-sedimented with the viral chromatin, while the rest of the T-Ag was found in the upper fractions sedimenting around 10–15S (data not shown).

A single site of T-Ag labelling is preferentially detected in the gap

Direct electron microscopic observations of minichromosomes reacted with antibodies against T-Ag failed to detect routinely the antibody molecules thus preventing clear identification of the site of interaction with the antigen. For these reasons we employed an indirect method for the detection of T-Ag. First, the T-Ag monoclonal antibodies (mouse IgGs) were reacted with the minichromosomes and then revealed on incubation with a solution of colloidal gold granules coated with anti-mouse IgG antibodies. The minichromosomes were

then separated from the free primary and secondary antibodies on a Sepharose CL 4B column. Finally, they were diluted and immediately spread. This process provided us with very clean preparations without unbound contaminating colloidal gold (Figure 1A). Low magnifications clearly revealed that only ~1–2% of the total minichromosomes were labelled. In addition, it is important to note that 12–15% of the population of minichromosomes displaying a gap also showed a T-Ag labelling which was always localized within the gap. The labelling, in the form of a dense mass covered with one or several colloidal gold granules, was always unique in a single morphologically defined mature minichromosome (Figure 1). A quantitative analysis carried out on 103 labelled molecules showed that 85 of them (84%) displayed a gap within which the labelling was localized almost exclusively near one of its borders. The other minichromosomes (16%), without gaps, were labelled on one of their DNA linkers (Fig-

Fig. 2. Association of T-Ag with one DNA-bound particle. A single T-Ag labelling per minichromosome appears associated with one DNA-bound particle (arrow) of variable size: 6 nm in **A**, 5 nm in **B**, 8 nm in **C** and 4 nm in **D**. This particle is localized on the gap (**B**) near one of its limits (**A**) or on an internucleosomal DNA linker (**C,D**) when no gap is present. Note that the distance between the colloidal gold granules and the DNA-bound particle in **A** is 19 nm. **A–C**: uranyl acetate staining; **D**: tungsten rotary shadowing; bar = 100 nm.

ure 2C,D). In these preparations, it was impossible to identify clearly labelled native replicating chromatin.

When larger immuno-complexes did not mask the underlying structures (Figure 2) an association of the antibody

against T-Ag with one DNA-bound particle displaying a variable size was revealed. This connection was confirmed by the distance between the DNA-bound particle and the most proximal colloidal gold granule which was always <20 nm,

Fig. 3. SV40 viral chromosomes displaying at least one DNA-bound particle on the nucleosome-free region (gap). On these spread nucleoprotein complexes a globular particle is observed on the DNA within the gap near one of its limits (arrow). In C, beside the globular particle a short segment (12 nm) of the naked DNA is irregularly thickened. **A:** uranyl acetate staining; **B,C:** tungsten rotary shadowing; bar = 100 nm.

or shorter than the theoretical length of two bound immunoglobulin G (IgG) molecules (24 nm).

The controls carried out with a normal serum and with rabbit antiserum against histone H2B confirmed the specificity of this indirect method. In the first control, no colloidal gold was observed on spread preparations, whereas anti-H2B labelling was detected exclusively over several nucleosomes and never in the gap (data not shown, Ghose *et al.*, 1983).

Observation of at least one globular particle bound to the gap region

Following uranyl acetate staining (Figure 2A–C and Figure 3A) of spread minichromosomes, the characteristic feature of 20–26 nucleosomes connected by linker segments of variable length was observed. 10–15% of the minichromosomes carried a nucleosome-free region extending over 200–400 bp. In addition, the presence of DNA-bound globular particles was observed in ~15% of these gaps, that is, in only 1–2% of the entire population, after staining alone or after staining followed by shadowing (Figure 3). These particles had a diameter between 4 and 8 nm and were in most cases observed as single particles localized near one limit within the gap. More rarely, up to three particles were present in the gap. They were separated and dispersed along the gap or closely juxtaposed, forming a short thickened fiber of 10–15 nm long (Figure 3C). In minichromosomes devoid of gaps a similar particle was sometimes observed bound to an internucleosomal DNA linker. These DNA-bound particles were similar to those detected with antibodies against T-Ag.

Mapping of the T-Ag labelling

The association between T-Ag labelling at the origin of DNA replication which is known to be present within the gap, was confirmed by the digestion of viral chromatin with three single-cut restriction endonucleases: *Bam*HI, *Eco*RI and *Bgl*II, either before or after labelling. In both cases, identical results were obtained. After *Bam*HI digestion, both the labelling and the gap were constantly localized in a central position of the linear molecules with an almost equal number of nucleosomes on both sides (Figure 4A–D).

In minichromosomes displaying a gap, *Eco*RI digestion resulted in an asymmetric nucleosomal pattern (Figure 4E). In this case, the labelling was preferentially localized within the gap close to the limit of the largest nucleosomal part of the molecule (Figure 4F). In the minichromosomes devoid of gaps, the colloidal gold was always in an asymmetric position (Figure 4G,H).

In the case of *Bgl*II, we noted that the efficiency of cleavages was reduced from 30% to 3–5% when only the population of antibody labelled minichromosomes was considered. Since, in this preparation, the proportion of labelled minichromosomes was similar to that (1–2%) found in undigested preparations, this reduction effectively represented a protection of the *Bgl*II site by the bound T-Ag in 80–90% of the minichromosomes containing T-Ag. In the *Bgl*II-linearized viral chromosomes that we observed, the gap was in a distal position. In this case, the labelling was found at the extremity of the gap (Figure 4I) in 23 out of 25 minichromosomes (90–95%) and was sometimes associated with a DNA-bound particle (Figure 4J). It was, however, rarely present at the other extremity of the molecule (on the early side of the *Bgl*II site) close to the first nucleosome (Figure 4K). When no gap was present the labelling was always observed at one extremity of linearized minichromosomes (Figure 4L).

Discussion

The interaction of the regulatory protein SV40 T-Ag with the SV40 minichromosome, allowed us to develop a very sensitive procedure for the specific detection of proteins interacting with native chromatin. We were able to identify a single binding domain for T-Ag on the SV40 chromatin. This domain is localized near to or at the origin of replication in minichromosomes containing or lacking a nucleosome-free region. In addition, an association between the T-Ag and a DNA-bound particle near the origin was postulated. Our extraction procedure led us to observe that only 1–2% of the total viral chromosomes were associated with immunologically reactive T-Ag. The very small fraction of the total T-Ag pool associated with morphologically defined mature minichromosomes probably corresponds to the T-Ag associated with SV40 form I DNA found by Mann and Hunter (1979) and to the tightly bound class observed by Reiser *et al.* (1980).

The correspondence between the labelling site of T-Ag and the origin of replication is demonstrated by T-Ag labelling preferentially associated within the gap near one of its borders and is confirmed by immunoelectron microscopic mapping after digestion by three restriction endonucleases. These experiments prove that the T-Ag tightly bound to chromatin has affinity for sequences at the origin of replication *in vivo* (Jessel *et al.*, 1976; Tjian, 1978; Shortle *et al.*, 1979; Shalloway *et al.*, 1980; McKay and Di Maio, 1981). The *Bgl*II site which is centered in T-Ag binding region II appeared

Fig. 4. T-Ag labelling of SV40 minichromosomes digested with single-cut restriction endonucleases. (A–D) after digestion with *Bam*HI. The gap is in a central position on the linearized nucleoprotein complex (A). In B, the T-Ag labelling is associated with the gap. When no gap is visible, the labelling is observed in the middle of the minichromosome (C,D). (E–H) after digestion with *Eco*RI. One observes an asymmetric repartition of the nucleosomes on both sides of the gap (E). The labelling is detected at the border of the gap near the largest nucleosomal region of the minichromosome (F). When no gap is present (G,H), the labelling is still observed in an asymmetric position. (I–L) after digestion with *Bgl*I. The cleavage site is very near one limit of the gap. The T-Ag labelling is always observed at one extremity of the linearized minichromosomes even when no gap is visible (L). When the distal gap is present, the labelling is localized either at the extremity of the gap (I,J) or at the other end of the minichromosome (K). In some cases, it is associated with one DNA-bound particle (J). Uranyl acetate staining; bar = 100 nm.

protected against cleavage when the minichromosomes were examined for the presence of T-Ag. This high level of protection could reflect the cooperative binding of T-Ag on at least regions I and II rather than binding on region II alone (Myers *et al.*, 1981b). Due to the very close proximity of sites I and

II, the structural configuration of the T-Ag bound only to the sites overlapping region I nearest the *Bgl*I site (Tegtmeyer *et al.*, 1983) might also protect against cleavage by *Bgl*I and explain the low proportion of linearized molecules labelled on the early side of the *Bgl*I site (region I). Concerning T-Ag

bound only to region III, it should be mentioned that Myers *et al.* (1981b) have shown that even in the absence of region I, T-Ag still binds specifically to regions II and III, albeit with much lower efficiency than in the presence of region I. Part of the observed labelled minichromosomes might be intermediates in the initiation of viral DNA synthesis (Mann and Hunter, 1979), although we cannot exclude an association of T-Ag with a very early replicating form (Tack and DePamphilis, 1983). Some of them might also correspond to repressed forms of early transcription (Hansen *et al.*, 1981; Myers *et al.*, 1981b; Rio and Tjian, 1983).

When the labelling did not mask the underlying chromatin, the detected T-Ag was always clearly associated with only one DNA-bound particle. Even on spread freshly-isolated minichromosomes two closely spaced particles were detected only rarely (Figure 3C). Similar particles observed near the origin of replication have been previously described (Saragosti *et al.*, 1980; Griffith *et al.*, 1975; Kasamatsu and Wu, 1976).

We established that the T-Ag labelling is resistant to treatment of minichromosomes with 1.0 M NaCl (data not shown). This result agrees well with the finding that association of SV40 T-Ag with the viral chromatin is salt resistant (Rudolph and Mann, 1983). As mentioned by these authors, the difference in salt resistance between the T-Ag bound *in vivo* and the soluble T-Ag reacting *in vitro* might be due to specific modifications of either T-Ag or DNA configuration. The very low proportion of labelled minichromosomes we have observed rather suggests the tight binding of a minor subclass of T-Ag to the viral DNA.

Two other possibilities could also account for the different *in vivo* and *in vitro* T-Ag-DNA interactions (Rudolph and Mann, 1983). First, other proteins might be associated with SV40 DNA in close proximity to bound T-Ag. This hypothesis seems unlikely since, in the present study, we very rarely observed two closely spaced particles bound to the origin of replication. The second possibility is an association specific or not of T-Ag itself with one or more other proteins. This agrees better with our observations of labelled DNA-bound particles which display variable sizes. However, we cannot exclude that the variability in size might be due to other possibilities such as the variable disaggregation of a homopolymer of T-Ag bound to the origin of replication. In conclusion, we wish to emphasize that our experimental procedure makes it possible to visualize proteins specifically bound *in vivo* and *in vitro* to native chromatin. In addition, it allows the direct observation of the spatial distribution of the proteins and their eventual association with substructures.

Materials and methods

Growth of cells and virus infection

African green monkey kidney cells (MA 134) were grown in Dulbecco's modified Eagle's medium supplemented with 7% fetal calf serum (Gibco) in 75 cm² plastic Petri dishes. Plaque-purified wild-type SV40 (0.5 ml/dish) was used to infect the cell cultures at a multiplicity of 50 p.f.u./cell. Two hours were allowed for adsorption, after which 10 ml medium containing 2% serum were added to each dish.

Isolation and partial purification of SV40 minichromosomes

MA 134 cells were labelled with [Me-³H]thymidine at 30 h post-infection. At 42 h, the minichromosomes were extracted by a modification of the method developed by Varshavsky *et al.* (1977).

The nuclear pellet obtained according to Varshavsky was suspended in 0.25% Triton X-100, 10 mM Na-EDTA, 0.10 M NaCl, 1 mM phenylmethylsulfonylfluoride (PMSF), 10 mM triethanolamine (TEA)-HCl, pH 7.3 and the suspension subjected to continuous very gentle stirring for 3 h at 4°C on a magnetic stirrer. The suspension was then centrifuged at 10 000 g for 2 min at

4°C and the supernatant layered on a linear 5–30% sucrose gradient containing 0.1 M ammonium acetate, 1 mM EDTA, 1 mM PMSF and 10 mM TEA-CH₃COOH (pH 7.3). The samples were centrifuged in a SW41 rotor at 40 000 r.p.m. and immediately used for the next step.

Digestion with restriction endonucleases

The digestion was carried out as described by Varshavsky *et al.* (1978) except for the final concentration of MgCl₂ which was adjusted to 5 mM. Restriction nucleases used in this work (*Eco*RI, *Bam*HI and *Bgl*II) were purchased from New England Biolabs.

Monoclonal antibodies and antiserum

Purified L16 monoclonal antibody against the SV40 large T-Ag and purified L19 monoclonal antibody against the SV40 T-Ag plus the SV40 small T-Ag were a kind gift from Dr. E. Harlow. These two mouse IgGs belong to the L series of monoclonal antibodies previously described (Harlow *et al.*, 1981). Control serum was a pool of sera obtained from normal adult mice.

The anti-SV40 tumor serum used was a pool of sera obtained from Syrian hamsters bearing tumors induced by inoculation of SV40-transformed EHSVi hamster cells. The corresponding control serum was a pool of sera obtained from 20 normal adult Syrian hamsters.

The rabbit antiserum against histone H₂B (chicken erythrocytes) was a kind gift of Dr. Van Regenmortel. The control serum was a pool of sera obtained from two adult rabbits.

Immunoprecipitation and gel electrophoresis analysis

Aliquots (200 µl) of cellular extracts of SV40-infected cells, extracted viral chromatin and some sucrose gradient fractions were immunoprecipitated overnight at 4°C with 1 µl of anti-T-Ag hamster serum. Conditions for immunoprecipitation and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) have been previously described (Kress *et al.*, 1979). Control incubations consisted of substituting normal hamster serum in place of immune serum.

Gels were stained by the silver staining method of Switzer *et al.* (1979).

Immunological reactions

We used the indirect colloidal gold method. Either 100 µl of the minichromosome pool or 30 µl of single-cut nucleoprotein complexes were mixed with a 1000-fold final dilution of anti-T-Ag monoclonal antibodies (mouse IgGs) overnight at 4°C. The mixture was then incubated at 37°C for 30 min with 1 µl of a solution of 5 nm colloidal gold granules coated with anti-mouse IgG (Janssen Pharmaceutica, Belgium). After incubation, the mixture was loaded on a 5 ml Sepharose CL 4B (Sigma, USA) column and eluted with the buffer used for the partial purification on sucrose gradients. The [³H]DNA peak was collected, diluted 5-fold with double-distilled water and processed immediately for electron microscopy. Control experiments were performed either with normal mouse serum instead of T-Ag monoclonal antibodies or with the solution of colloidal gold anti-mouse IgG alone.

As the antigenic motifs of histone H₂B are found at the periphery of the core particle (Absolom and Van Regenmortel, 1978) and is the most accessible histone (Goldblatt *et al.*, 1978), a control for the specificity of this immunodetection method was carried out the same way as above with anti-H₂B serum (rabbit IgG) diluted 100- to 1000-fold and then with 20 nm colloidal gold granules coated with anti-rabbit IgG (Janssen Pharmaceutica, Belgium).

Electron microscopy

The intact viral complexes and the restriction nuclease-digested ones were diluted 10- to 20-fold and 3- to 5-fold, respectively, with double-distilled water and 5 µl were absorbed for 90 s to electron microscopy grids coated with a positively charged carbon film (Dubochet *et al.*, 1971). Samples were stained with 2% (w/v) aqueous uranyl acetate and rotary shadowed with tungsten under an angle of 8°. They were observed in a Philips 400 electron microscope operated at 80 kV.

All the measurements were carried out on positive prints at a final magnification of 240 000–800 000. The length measurements were performed with a Hewlett Packard graphic digitizer. Diameters of particles and nucleosomes were measured using a precision magnifier (Nachet, France).

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