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Herpes specific and α DNA polymerase in nuclear envelope of BHK infected cells

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SUMMARY

Nuclear envelopes of normal and Herpes simplex virus infected BHK cells were isolated and separated in two fractions by a step gradient procedure. α, β and Herpes-specific DNA polymerases activity were tested in the chromatin and nuclear envelope fractions of these cells. We found that both α and Herpes-specific DNA polymerases activity were associated with only one of the two nuclear envelope fractions and were released by Triton X-100 treatment. On the contrary, β DNA polymerase activity was associated with the chromatin fraction. More endonucleotylic activity and particularly apurinic and UV specific endonucleases were found associated with the same nuclear envelope fraction. These findings suggest an active role of the nuclear envelope in the replication of both cellular and Herpes simplex viral DNA.

INTRODUCTION

Nuclear envelope surface enhancement of Herpes infected BHK cells is the most striking morphological effect observable early after viral infection (1,2). One can ask as wether this multiplication correspond to a functionnal state necessary to virus production. Independently of the role of the nuclear membrane in the envelopment of the virus from the inner leaflet (3) it was tempting to test it for specific Herpes DNA polymerase activity. This enzyme is virus coded but not included in the virion and previous work suggests that virus DNA replication occured at or near the nuclear boundaries (2).

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Presence of DNA polymerases as a transient constituent of the nuclear envelope or nuclear matrix of actively replicating nuclei is strongly suggested both by the work of Pardoll et al. (4) and Smith and Berezney (5). The first showed that the newly replicating complexes were likely to be bound to the nuclear matrix while the second demonstrated that in regenerating liver the α DNA polymerase activity was copurifying with nuclear matrix as compared to resting liver where it was located at the chromatin level.

In this work we tested for both α, β and Herpes-specific DNA polymerase in inner and outer nuclear membrane fractions purified on discontinuous sucrose gradient following the technique of Kasper (6). We correlate these activities and both thymidine kinase and nucleolytic activity in control and Herpes infected BHK cells.

MATERIALS AND METHODS

Cells:

BHK cells were grown at 37°C in α medium (Gibco) supplemented with 10% fetal calf serum (Flow Laboratory, mycoplasma-free) and 1% kanamycin (Gibco). Herpes simplex virus type 1 (HSV-1) tsG8 strain (from Dr. P. Schaffer) was propagated and assayed on BHK cells. Confluent monolayers of BHK cells were infected in petri dishes with 2 plaque forming units (PFU) per cell and incubated at 37°C for 45 min. After infection, the cells were incubated 12 hours at 31°C in α medium containing 2% fetal calf serum and 1% kanamycin.

Preparation of nuclear envelope:

The technique used is basically as described by Kasper (6) modified to fit the buoyant density of BHK nuclear membrane. Cells are carefully washed with PBS, resuspended in 0.25 M sucrose containing 50 mM Tris pH 7.5, 5 mM $MgCl_2$ and 2.5 mM KCl (TKM) and homogeneised in a potter homogeneiser. When cells are clearly broken as seen in a phase microscope, the suspension is made 1.2 M in sucrose, layered after careful mixing on a 10 ml cushion of 1.8 M sucrose in TKM and centrifuged for 70 minutes at 104,000 g at 4°C. The nuclear fraction is then in the form of a pellet while most of the cytoplasmic constituents stay above the sucrose cushion.

Nuclear pellets are resuspended in 15 ml 1 M sucrose in TKM by gentle pipettation and centrifuged at 3,400 g in a Sorval centrifuge. The nuclei are resuspended in 0.25 M sucrose in TKM and centrifuged at 750 g for 10 minutes. The nuclear fraction is then sonicated at half scale in a sonifier MSI while nuclear breaking is monitored by phase microscopy. Sonication is stopped when nuclear envelopes are still visible. To the sonified extract, 2 ml of a 60% potassium citrate solution in TKM is added and mixed for 1 to 2 minutes. This mixture is centrifuged at 39,000 g. The semi-gelatineous pellet is constituted by nuclear membranes and contaminants while the supernatant represent the cells chromatin.

The nuclear membrane pellet is processed as in Kasper (6) but for the sucrose concentrations which are respectively 26.4 g/100 ml, 31.3 g/100 ml

and 37 g/100 ml, in three layers of equal volume. This multilayered gradient is centrifuged at 120,000 g for 14 hours. The nuclear membranes are found in two bands between the two first layers and between the second and the third (see results).

Nuclear membrane bands are either processed for electron microscopy or SDS-polyacrylamide gel electrophoresis or resuspended in 1% Triton X-100 in TKM using a Teflon-glass homogeneiser. After a 20,000 g centrifugation for 30 minutes the supernatant is assayed for enzymatic activities.

Assay for DNA polymerases activity:

Reaction mixture for α and β polymerases are essentially following Falaschi and Spadasi (7). The α DNA polymerase mix contained 20 mM K phosphate, pH 7.2, 0.1 mM EDTA, 0.5 mM DTT, 10 mM $MgCl_2$, 250 µg/ml BSA and 200 µg/ml activated DNA with all four dNTP at 500 µM each, to which [^{3}H]-TTP is added (43 mC/mM).

The β DNA polymerase mix contained 50 mM Tris-HCl pH 8.5, 0.1 M KCl, 10 mM $MgCl_2$, 1 mM DTT, 250 µg/ml BSA and 200 µg/ml of activated DNA, all four dNTP at 500 µM each and [^{3}H]-TTP. To this reaction mix, 5 mM N-ethylmaleimide is added to block α DNA polymerase activity (7).

For Herpes specific polymerase, the reaction mixture contained 20 mM Tris-HCl pH 7.5, 8 mM $MgCl_2$, 0.4 mM EDTA, 5 mM β-mercaptoethanol, 200 mM KCl and 200 µM of each dNTP to which [^{3}H]-TTP is added. The assay is done using 60 µg/ml of single stranded DNA (8). For these three assays, mixtures are incubated for 45 minutes at 37°C, TCA precipitated and counted on filters.

Assays for nucleases and thymidine kinase activity:

The substrates consist of [^{3}H]-labeled ØX 174 either in a closed supercoiled replicative form (double stranded specific endonuclease), single stranded form (single stranded specific endonuclease), open form (exonuclease), or acid pH treated replicative form (AP endonuclease) (9) and were a gift of Dr. L. Grossman. Thymidine kinase was assayed following Hay et al. (8).

Protein determination:

The amount of protein used for each assay was determined using the Biorad protein assay standardised for a buffer containing 1% Triton X-100.

RESULTS

For both infected and non infected cells, two clear bands containing membrane fractions were seen on the step wise gradient. An electron microscopic study of these bands shows that while there is more than just nuclear membranes in the two fractions from infected cells (fraction Inf and Sup) there are more contaminations by both mitochondrion and viral particles in fraction "Sup". A detailed structural study of the nuclear membrane fractions and their purification will be published elsewhere. In the Triton X-100 extract of nuclear membrane from infected cells one can see a characteristic band at around 150,000 MW and another one at around 120,000 MW which are strengthened during purification. A good idea of the separation obtained through these

Table 1. DNA polymerases activity in different fractions

α DNA polymerase, β DNA polymerase and Herpes specific DNA polymerases were assayed as described in Materials and Methods. Results are presented in net cpm after background (50 cpm) substraction. An identical protein concentration was used for assay of each fraction. As one can see both α DNA polymerase and Herpes DNA polymerase is more active in the inferior band of nuclear membrane as compared to both superior band and chromatin fraction. The inverse is observed for β DNA polymerase.

		α Polymerase (net cpm)	β Polymerase (net cpm)	Herpes-specific DNA Pol (net cpm)
Chromatin		40	1680	—
Chromatin infected		50	3129	46
Nuclear membrane Non infected	sup	753	110	—
	inf	4100	220	—
infected	sup	415	150	660
	inf	1414	230	16850

steps is also given by the actual protein concentration of each sample which shows grossly a purification factor of 10 for each step.

Table 1 shows the difference of activity found in nuclear membrane and in chromatin for the three different DNA polymerases tested.

As can be seen from this table, β polymerase is mostly situated in the chromatin part of the nuclei while α polymerase is clearly associated with the nuclear envelope and extracted by Triton X-100. The inferior band of the sucrose gradient is the richest in both α polymerase and herpes specific DNA polymerase which is the most active of all and clearly associated to the nuclear envelope.

The nucleolytic activity of the different fractions is illustrated in Table 2.

It is clear from this table that the nuclear membrane is richer in Ap endonuclease and UV specific endonuclease in infected cells than in control cells. The level of non specific endonuclease however is similar in both and exonucleases activity are very low both on single stranded and double stranded DNA.

Table 2. Nucleolytic activities in nuclear envelope fractions

Nucleolytic activities on substrate consisting into single-stranded (SS), double-stranded (DS), UV-treated and acid-treated DNA were assayed as described in Materials and Methods. An identical amount of proteins was assayed for each test. There is a clear enhancement of Ap endonuclease (Ap endo) and UV specific endonuclease (UV endo) in nuclear membrane of infected cells as compared to non specific endonuclease (non specific endo).

Nuclear membrane	Ap endo	Ap endo - non specific endo	UV endo	UV endo - non specific endo	Non specific Endo	Exonucleases	
						SS	DS
Non infected	10516	6416	5462	1362	4100	231	53
Infected	23775	17890	12081	6136	5885	179	38
Total counts	30000		23000		24000	1280	17000

The HSV-specific thymidine kinase activity was also measured on the chromatin and the nuclear membrane fractions as show in Table 3.

We can see that the inferior nuclear membrane is still fairly active in the infected cells fractions even if the HSV-specific thymidine kinase is mostly located in the chromatin.

DISCUSSION

Electron microscopic evidence of ontogenesis of Herpes simplex viral particles at the nuclear envelope locus have been described by many groups

Table 3. Herpes-specific thymidine kinase in different fractions

HSV-specific thymidine kinase activity was assayed as described in Materials and Methods. Results are expressed in net cpm after background subtraction (890 cpm).

The enzymatic activity is mostly located in the chromatin fraction but the inferior band of nuclear membrane is still fairly active.

	Herpes-specific thymidine kinase	
	Normal cells (net cpm)	Infected cells (net cpm)
Chromatin	—	16,562
Nuclear membrane		
Sup	—	400
Inf	—	1,504

(1,10,11). Autoradiographic studies of viral nucleic acid synthesis have shown that the newly synthesized DNA is mostly located near the nuclear envelope of infected cells (2). On the other hand, current models of eukaryotic DNA synthesis (4,5) implies the transient presence of the DNA replicating enzyme at the nuclear matrix level. It was of logic in the case of Herpes simplex virus to look for its replicative enzyme at this location. Indeed the presence of both α DNA polymerase which is responsible for cell DNA replication and Herpes specific DNA polymerase in the inner nuclear membrane fraction strengthen the hypothesis of an involvement of the nuclear envelope in cellular or viral DNA replication. It is to be noted here that in some experiments with HSV-infected BHK cells, some DNA polymerase activities were also found in the chromatin fraction. This can be a time directed phenomena where different enzymes are situated in different cellular compartments and constitutes in itself an interesting phenomena on which we are currently working.

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