Tick-borne encephalitis virus-specified sequences in persistently infected cell culture revealed by DNA-DNA hybridization

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Summary. Hybridization of DNA probe, obtained through DNA polymerase-mediated in vitro transcription of tick-borne encephalitis virus (TBEV) RNA, with DNA isolated from persistently infected with TBEV cell culture revealed 5.4 copies of viral genome per haploid set.

One of the most prominent problems of medical virology is a particular category of human and animal diseases due to long-term persistence of viruses. Chronic viral infections entail pronounced deterioration of several cell systems and affect the cellular genetic apparatus. It has been demonstrated previously by RNA-DNA hybridization experiments that the genome of several RNA viruses might be incorporated into the host cell genome in long-term persistent infection of cell cultures[2-4]. RNA-DNA hybridization technique is widely used for detection of homology between different types of nucleic acids, but possesses an essential disadvantage, that is, DNA strand reassociation appears to be a more rapid process than RNA-DNA hybridization[5]. Due to simultaneous DNA reassociation, double-stranded DNA fragments are formed that include virus-specific sequences. As a result, a much lower number of viral genome DNA copies is detected by RNA-DNA hybridization technique.

Exploration of the molecular biology of chronic viral infections requires further improvement of molecular hybridization technique for detection of virus-specific sequences in cellular DNAs. Hybridization of cellular DNAs with DNA-probes synthesised in vitro along a viral RNA template represents a more adequate technique, when data concerning the number of incorporated viral genome copies are required[6]. A well-known mode of synthesis of genetic material with the ordered base sequence is the reverse transcription that utilizes the viral enzyme reverse transcriptase. Recently DNA-polymerases were used to obtain DNA copies of ribosomal RNA[7]. In this work we have made an attempt to obtain DNA-transcript of tick-borne encephalitis virus (TBEV) RNA with the aid of *Escherichia coli* DNA-polymerase I, and to determine the presence of virus-specific sequences in DNA preparations from persistently infected cell culture by DNA-DNA hybridization.

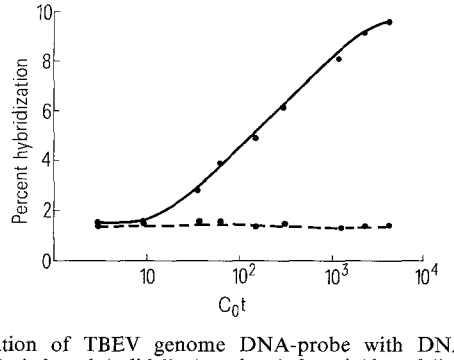
Determination of the reverse transcriptional activity of *E. coli* DNA-polymerase I was carried out utilizing a synthetic template with poly A·oligo dT as a primer. The enzyme concentration and incubation conditions were determined experimentally. Maximal output of DNA-product was observed with 30 units of enzyme and 25 μg poly A·oligo dT per 1 ml of reaction mixture (50 mM tris-HCl, pH 7.9; 10 mM $MgCl_2$); 0.5 mM of dATP, dGTP, dCTP, each, and 0.02 mM ^3H TTP (100 counts/min/pM), when incubated for 1 h at 37 °C.

In vitro synthesis of DNA-probe of TBEV RNA was carried out with 30 units of *E. coli* DNA-polymerase I along RNA template (20 μg) without synthetic polynucleotides. The DNA-polymerase and the template were incubated in 1 ml of standard reaction mixture at 37 °C for 1 h. The DNA product was extracted with chloroform-isoamyl alcohol mixture, ethanol precipitated and dissolved in 0.4 M NaOH. RNA was degraded by 4 h hydrolysis at 45 °C. The DNA product so far obtained sedimented in alkaline sucrose gradients by a broad peak with a sedimentation coefficient of 6S. Hybridization of DNA-product with excess TBEV RNA in 5× SSC-0.5% SDS resulted in 90% resistance to S1 nuclease digest. For hybridization assays, DNA-probe and cell DNA were taken in proportion of $1:10^6$. Preincubation of DNA mixture in 0.1 M CH_3COONa, pH 4.2, for 50 min at 70 °C, followed by addition of 0.25 volume of IN NaOH and further incubation for 20 min at 100 °C results in additional hydrolysis of RNA and shearing of DNA into homogenous pieces[7]. Ethanol precipitated material was resuspended in hybridization buffer (0.6 M NaCl; 0.02 M tris, pH 7.4; 0.01 M EDTA; 0.05% SDS) at DNA concentration of 6 mg/ml, heated at 100 °C and transferred to 68 °C. Aliquotes were taken at appropriate times and treated with S1 nuclease.

The combined data on a series of DNA-DNA hybridization experiments are presented in the figure in accepted coordinate system – C_0t (DNA concentration in moles/l per hybridization time in sec) vs percent of hybridization. The figure demonstrates a noticeable level of DNA-probe hybridization with DNA from persistently infected cells, whereas hybridization with DNA from uninfected cells is virtually at the background level.

Calculation of the DNA-copy number incorporated, based on comparison of experimental kinetic curve with a theoretical second order reaction curve with certain parameters, allowed us to determine the degree of genomes integration, equal to 5.4 copies of viral genome per haploid set of persistently infected cell.

The results of this work indicate a relatively low content of TBEV-specified sequences in DNA from persistently infected cells. Integration of a small number of viral genome copies, while affirming the probability of DNA provirus formation, cannot clearly account for the phenomenon of viral persistence on the whole. Another question concerning conservation of the usual way of virus-specific RNA reproduction in TBEV persistently infected cell culture, may elucidate the role of 'integrated provirus' in the course of persistent virus infection.



Hybridization of TBEV genome DNA-probe with DNA from persistently infected (solid line) and uninfected (dotted line) cells. Abscissa: C_0t (DNA concentration in moles/l per hybridization time in sec). Ordinate: Percent of hybridization.

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A simple one-step procedure for staining the nucleolus organizer regions

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Summary. A simple silver staining technique for routine use is described by which the nucleolus organizer regions of mammalian chromosomes, including those of mouse chromosomes, are stained selectively.

Using the Ag-As staining technique developed by Bloom and Goodpasture[2], it is possible preferentially to stain rRNA genes in animal and plant cells. Miller et al.[3] have shown that a positive result of the staining is at the same time an indication of the activity of these genes.

For staining purposes, the two-step procedure (Ag-As) of Bloom and Goodpasture[2] is now used predominantly, in which first silver nitrate solution and subsequently ammoniacal silver solution and formalin are used as developers. One difficulty of this method is the control of the short developing time, whereby an overstaining often occurs.

An easier technique to stain the nucleolus organizer regions, the single application of silver nitrate solution, has already been mentioned by the authors[2]. The staining quality is excellent, the reproducibility, however, is very poor. The cause of this appears to be that the storage of the $AgNO_3$-solution (either in the dark or in the light, at room temperature or cooled, as well as the age of the solution) is decisive for the routine use of this technique.

When these parameters are controlled, the staining time can be accurately predicted after a single testing of the solution. The $AgNO_3$-solution cannot be used for an unlimited time. Storage in a dark-brown bottle at +4 °C in a refrigerator has proved best. It appears that the developing time decreases with increasing age of the silver nitrate solution. When using a freshly prepared Ag-solution (1–2 days old), the Ag-bands occur after 24–48 h. This period is reduced to 4–5 h, when a 3–5-week-old silver nitrate solution is used (figure 1). After more than 6–8 weeks, the solution no longer stains the NORs preferentially. If the silver nitrate solution is stored in clear bottles in the light at room temperature, a premature ageing process sets in which leads to a shorter developing time.

The age of the preparations (some days or months) and the storage temperature (+4 °C or +20 °C) are virtually of no significance for the staining process.

The individual steps are as follows:

1. Preparation of air-dried chromosomal preparations as usual.
2. Application of 50% w/v silver nitrate solution ($AgNO_3$ in aqua bidist.) and mounting. An air-tight closing of the cover-glass with cover-glass cement, the melting point of

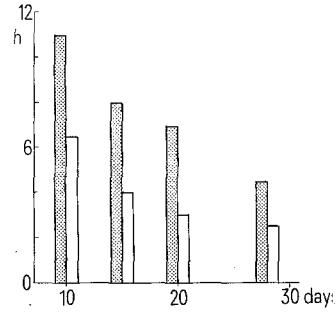

Fig. 1. Dependence of the staining time on the age of the silver nitrate solution when the solution is stored in a dark-brown bottle (hatched columns) and in a clear glass bottle.

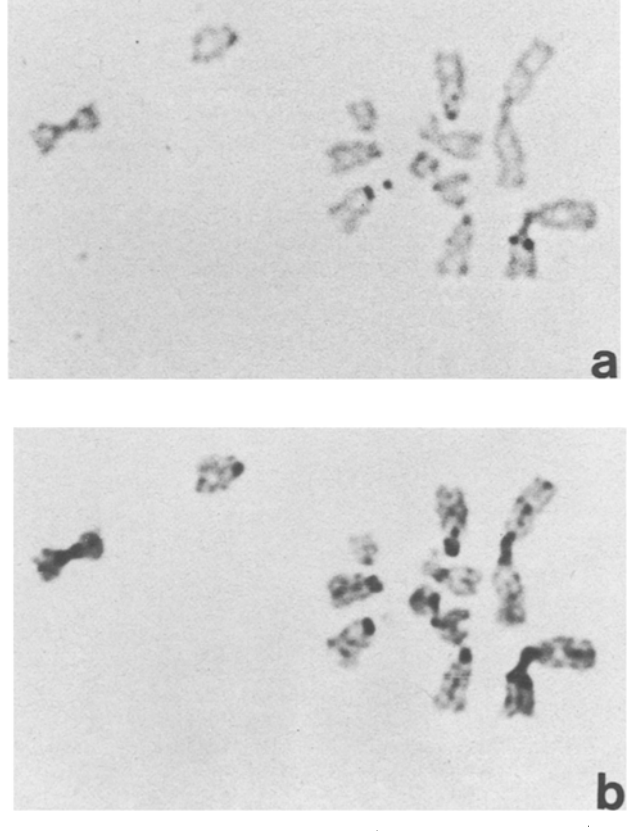

Fig. 2. Silver stained metaphase chromosomes of the mouse A-9 cell line. At first the NORs are selectively stained (a), after prolonged staining the C-band regions also became visible (b).