De Novo Synthesis of Two Classes of DNA Induced by Vaccinia Virus Infection of HeLa Cells

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SUMMARY

Equilibrium density gradient centrifugation in CsCl confirms that DNA synthesized after vaccinia virus infection of HeLa cells is homogeneous in buoyant density and thus in base composition and is similar in this respect to bulk HeLa cell DNA. In contrast, rate sedimentation in alkaline sucrose gradients distinguishes two main classes of virus-induced DNA, neither of which can be equated with cell DNA synthesized in the same cultures prior to infection. The slower sedimenting class of virus-induced DNA co-sediments with DNA from purified virus particles: the second class sediments faster than pre-labelled cell DNA. Heterogeneity of virus-induced DNA does not result from fragmentation of radioactively labelled DNA, virus-mediated breakdown of cell DNA or association with either proteins or polyamines. Both slow and fast sedimenting classes of virus-induced DNA contain sequences complementary to all restriction endonuclease HindIII-specific fragments of the virus genome. The multiple species of DNA synthesized after infection are distinguished further by the effect of ethidium bromide. At a concentration which prevents the formation of infectious progeny virus, this compound inhibits selectively the de novo synthesis of that class of virus-induced DNA which sediments faster in alkaline sucrose gradients.

The vaccinia virus genome has been shown to be a linear duplex DNA molecule for which mol. wt. estimates vary from $170 \times 10^6$ (Sarov & Becker, 1967) to $120 \times 10^6$ (Berns & Silverman, 1970). The G+C content of vaccinia virus DNA is sufficiently similar to that of mammalian cell DNA to make separation by isopycnic centrifugation impractical except after denaturation and selective reannealing (Jungwirth & Dawid, 1967). Virus DNA has been shown to be present in cytoplasmic aggregates in vaccinia-infected cells (Cairns, 1960; Loh & Riggs, 1961; Harford et al. 1966) and several studies of virus DNA replication have been made using cytoplasmic fractions of such cells. Joklik & Becker (1964) observed that newly replicated virus DNA was associated with large aggregates which sedimented rapidly in neutral sucrose gradients although their stability was dependent on the presence of Mg$^{2+}$ ions. These aggregates have been shown to contain both replicated virus DNA and protein (Dahl & Kates, 1970a) and to be capable of virus-specific mRNA synthesis in vitro (Dahl & Kates, 1970b). Rapid sedimentation of such structures under these conditions appears to result from association of DNA with virus-specific proteins and treatment with pronase of SDS releases DNA having sedimentation characteristics similar to those of virion DNA in neutral sucrose gradients (Polisky & Kates, 1972). However, virus-induced and cell-specific DNA species can be separated easily by lysis of whole, infected cells and rate zonal centrifugation in alkaline sucrose gradients in which denatured DNA sediments in a random coil configuration at velocities proportional to the lengths of the molecules. Anomalous sedimentation behaviour in denaturing gradients and electron microscopic observations indicate that the complementary strands of the vaccinia virus
genome are cross-linked covalently at or very near the termini (Geshelin & Berns, 1974). The present report describes two main classes of vaccinia-induced DNA detected in denaturing gradients. Neither of these is related to cell DNA synthesized in the same cells prior to infection.

The Lister strain of vaccinia was pock-purified by growth on the chorioallantoic membrane of fertile hens eggs and the morphology of virus particles was checked by electron microscopy. Vaccinia virus was propagated, purified and used to infect HeLa cell monolayers essentially as described previously (Joklik, 1962; Williamson & Archard, 1976). Stock cell cultures were propagated routinely in the absence of antibiotics. Media used for experimental cultures contained 200 μg/ml kanamycin, 100 μg/ml streptomycin and 100 units/ml penicillin.

Infected or sham-infected HeLa cell monolayers were labelled in the DNA by incubation from 2 to 4 h p.i. in medium containing 0.2 μCi/ml 2-14C-thymidine (62 mCi/mmol) or 1.0 μCi/ml 6-3H-thymidine (5000 mCi/mmol) respectively. Most progeny vaccinia DNA is synthesized during this period (Joklik & Becker, 1964). The cells were washed and recovered by treatment with 0.02% EDTA in phosphate buffered saline solution (PBS A), pooled and digested for 3 h at 37 °C with 0.1% sodium n-lauroyl sarcosinate (Sarcosyl NL 35), 0.1% pre-incubated pronase and 0.02 M-EDTA prepared in 0.015 M-sodium chloride, 0.0015 M-sodium citrate, pH 7.3. The digests were dialysed overnight against similar buffer and diluted 1/50 into 60% (w/v) caesium chloride solution to give a final density of 1.76 g/ml. Aliquots of 4.5 ml were centrifuged at 18 °C and 120,000 g for 60 h and the resulting gradients fractionated by upward displacement. After the addition of 1 mg calf thymus DNA as carrier, fractions were examined for acid-precipitable radioactivity by liquid scintillation spectrometry essentially as described previously (Archard & Williamson, 1971). Both 14C-labelled DNA from infected cells and 3H-labelled DNA from uninfected cells banded as single peaks. The peaks were almost coincident, having buoyant densities of about 1.7 g/ml (data not presented). These results are in agreement with the determinations of Jungwirth & Dawid (1967) and confirm that vaccinia virus-induced DNA is homogeneous in overall base composition and is similar in this respect to HeLa cell DNA.

DNA was analysed in further experiments by rate sedimentation in 5 to 25% (w/v) alkaline sucrose gradients. Gradients were prepared in polypropylene tubes (boiled previously for 1 h in 0.001 N-NaOH, 0.1 M-EDTA) by layering 2.2 ml vol. of sucrose solutions in 0.7 M-NaCl, 0.005 M-EDTA, 0.3 N-NaOH (gradient vol. 11 ml) and allowed to diffuse for 3 h at room temperature. HeLa cell monolayers, pre-labelled in the DNA by incubation for 16 h in medium containing 0.25 μCi/ml 6-3H-thymidine, were washed twice and re-incubated for 2 h in medium lacking the labelled precursor to reduce unincorporated radioactivity. The cultures were then infected, virus-induced DNA labelled by incubation from 2 to 4 h p.i. in medium containing 0.25 μCi/ml 2-14C-thymidine and the cells recovered as before.Volumes of 0.5 ml of 1 N-NaOH were floated on the gradients and 0.5 ml samples of cell suspension containing about 3 × 10^6 cells and 0.02 M-EDTA were layered and the cells allowed to lyse in situ for 30 min. The gradients were centrifuged at 18 °C and 110,000 g for 2 h, fractionated by upward displacement and the incorporated radioactivity determined as described (Fig. 1 a). DNA synthesized before infection and labelled with 3H-thymidine was isolated as a single major peak. In contrast, DNA labelled in the same cells with 14C-thymidine during the period of maximum virus DNA synthesis was isolated as two, widely separated, major peaks. One peak sedimented faster and the other more slowly than the 3H-labelled, cell DNA. Additionally, there was evidence of minor peaks of 14C-labelled DNA sedimenting at intermediate velocities and possibly coincident with cell DNA. These results
show that DNA synthesized in infected cells during the period of maximum virus DNA synthesis and homogeneous on the basis of equilibrium buoyant density may be resolved as two major fractions by rate sedimentation. Neither of these is coincident with HeLa cell DNA labelled before infection. Apparent, multiple species of DNA detected after infection do not result from either degradation or re-utilization of pre-labelled, cell DNA (Parkhurst et al. 1973) as only the labelled thymidine supplied and incorporated during the period of virus DNA synthesis is found at these positions in the gradients. Additionally, such heterogeneity does not result from spontaneous fragmentation of $^3$H-labelled DNA (Peterson & Fox, 1971) as similar sedimentation profiles are obtained when the labelling is reversed. In additional experiments, vaccinia-infected HeLa cell monolayers were labelled from 2 to 4 h.p.i. with $^3$H-thymidine ($2\cdot0 \mu$Ci/ml) plus either $^{14}$C-leucine ($62 \text{ mCi/mmol, } 0\cdot5 \mu$Ci/ml) or $^{14}$C-spermidine ($122 \text{ mCi/mmol, } 0\cdot5 \mu$Ci/ml) and analysed in denaturing sucrose gradients as before. Radioactivity supplied as either $^{14}$C-leucine or $^{14}$C-spermidine did not co-sediment with incorporated $^3$H-thymidine but remained near the top of the gradients (data not presented). These results indicate that the sedimentation characteristics of virus-induced
DNA species from vaccinia-infected cells do not result from association with either proteins or polyamines. Vaccinia virus grown in the presence of $^3$H-thymidine, purified by isopycnic centrifugation in potassium tartrate density gradients and analysed under similar conditions yielded DNA co-sedimenting with the slower sedimenting DNA induced in HeLa cells by virus infection. The peak position of T₄ bacteriophage DNA labelled with either $^3$H-thymidine or $^{32}$P-orthophosphate is indicated by the superimposed arrow. Denatured DNA from vaccinia virus particles sediments often but not invariably in alkaline gradients as a bifurcated peak. If the mol. wt. of T₄ DNA is taken as $130 \times 10^6$ with a sedimentation coefficient of about 70S in denaturing gradients (Studier, 1965) then the major component of DNA from isolated vaccinia virus particles sediments at 98S representing a single strand of $150 \times 10^6$ mol. wt. The minor component presumably represents a single stranded circle of the same molecular weight as the cross-linked, native genome from which the 98S form is generated in denaturing conditions by a single nick (Geshelin & Berns, 1974).

The specificity of DNA sedimenting at various positions in denaturing gradients was determined by hybridization to restriction endonuclease Hind III-specific fragments of vaccinia virus genome DNA, prepared essentially as described by Müller et al. (1978), and of
HeLa cell DNA. Restriction fragments were separated by electrophoresis in 0.6% agarose slab gels, denatured and transferred to nitrocellulose membrane filters (Southern, 1975). Fractions containing the slow and fast sedimenting peaks of virus-induced DNA from infected cells were taken from gradients similar to that described in Fig. 1(a) and the DNA recovered by ethanol precipitation after neutralization with HCl in the presence of 100 mM-tris-HCl, pH 7.4. DNA was labelled by nick translation essentially as described by Rigby et al. (1977). In addition to the recovered DNA, reaction mixtures (100 µl) contained 20 µM-deoxyribonucleotide triphosphates including 2-5 µCi each of (α32P)-d ATP and (α32P)-d CTP (specific activities 150 Ci/mmol), 50 mM-tris-HCl, pH 7.4, 5 mM-MgCl2, 1 mM-β-mercaptoethanol, 5 µg BSA and 2 units of DNA polymerase I (Boehringer Corp.); DNase was omitted. Nick translation was at 14 °C for 2 h and DNA was re-isolated by exclusion chromatography on Sephadex G50 followed by ethanol precipitation. Labelled DNA was denatured at 105 °C for 5 min and 20000 to 50000 Cerenkov ct/min were hybridized for 60 h to representative nitrocellulose strips bearing transferred, restricted vaccinia virus or HeLa cell DNA. Pre-treatment of strips, conditions of hybridization and subsequent washing were similar to those described by Jeffreys & Havell (1977). Strips were autoradiographed for 3 to 10 days at −70 °C using pre-sensitized Fuji Rx X-ray film and an Ilford fast tungstate intensifying screen. DNA recovered from gradient fractions containing either the slow or fast sedimenting peak of virus-induced DNA from infected cells hybridized specifically to all Hind III restriction fragments of known vaccinia virus DNA to which control, nick translated, vaccinia virus total DNA hybridized also (Fig. 2a). Control 32p-labelled HeLa cell DNA failed to hybridize to vaccinia DNA under these conditions. These results indicate that DNA synthesized during the period of maximum virus DNA replication in vaccinia-infected cells and either co-sedimenting with the vaccinia genome or sedimenting faster than in vivo pre-labelled cell DNA contains sequences complementary to all Hind III restriction fragments of the virus genome. In contrast, such DNA failed to hybridize strongly to Hind III restricted HeLa cell DNA demonstrating that both the slow and fast sedimenting peaks of DNA induced by virus infection consist mainly of virus-specific sequences (Fig. 2b).

In further experiments, infected HeLa cell monolayers were maintained from 1 h p.i. in media containing various concentrations of ethidium bromide. From 2 to 4 h p.i., the media also contained 0.25 µCi/ml 14C-thymidine. The cells were recovered and washed and samples analysed in alkaline sucrose gradients as before (Fig. 1b). In the presence of 10 µg/ml ethidium bromide, the sedimentation profile of virus-induced DNA was similar to that found with non-inhibited, infected cells. In the presence of 20 µg/ml or 30 µg/ml ethidium bromide, the amount of incorporated radioactivity present in the faster sedimenting peak was inhibited markedly while that in the slower sedimenting peak was relatively unaffected. At an inhibitor concentration of 50 µg/ml, the incorporation of radioactivity into the faster sedimenting peak was inhibited almost completely. These results confirm that two classes of DNA, other than cell DNA, are synthesized after infection and indicate that the synthesis of one such class is particularly sensitive to inhibition by ethidium bromide. This intercalating agent is preferentially bound by circular DNA (Radloff et al. 1967) and has been shown previously to inhibit the growth of vaccinia virus (Vilaginés, 1970) or the replication of DNA in cytoplasmic fractions of vaccinia-infected cells (Raya & Vilaginés, 1970). In the present study, the presence of 20 µg/ml ethidium bromide from 1 h p.i. completely inhibited the production of infectious progeny virus in vaccinia-infected HeLa cells (data not presented) suggesting that the fast sedimenting form of virus-induced DNA observed in denaturing gradients is essential to virus replication.
As no evidence was obtained for association of protein with the fast sedimenting material it seems unlikely that this is equivalent to the large aggregates observed previously in neutral gradients (Dahl & Kates, 1970a) or that pronase treatment could result in conversion to a form co-sedimenting with the virus genome (Polisky & Kates, 1972). Hybridization data suggest strongly that fast sedimenting DNA induced by vaccinia infection consists mainly of virus-specific sequences. The presence of some cell-specific sequences cannot be excluded although the material failed to hybridize strongly to HeLa cell DNA. However, degradation of cell-specific, nascent DNA by a virion nuclease leading to inhibition of host nuclear DNA synthesis after vaccinia infection (Pogo & Dales, 1974) makes it unlikely that sedimentation characteristics result from association of virus DNA with very high mol. wt. cell DNA. The separation described depends on the size and conformation of DNA molecules rather than their density. The slower sedimenting peak from infected cells appears to correspond to the virus genome and the faster-sedimenting peak to a class of virus-induced DNA which is larger or whose conformation, resistant to alkaline denaturation, favours rapid sedimentation and preferential binding of ethidium bromide. The sedimentation behaviour of vaccinia virus genome DNA in denaturing conditions is anomalous as the complementary strands of the duplex are linked covalently in a manner reminiscent of a continuous single strand polynucleotide chain. Nick translation of fast sedimenting material from denaturing gradients yields labelled DNA sequences representative of the total virus genome suggesting that, in this case also, strand separation is not complete and that rapid renaturation occurs after neutralization. It is suggested that the fast sedimenting material described here may be a partially circular replication complex of this unusual virus genome.

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REFERENCES


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