DNA into the cellular genome, and analysis of SV40 DNA insertions may predict the fate of other exogenous DNAs. Although cells may incorporate exogenous DNA efficiently, that DNA is unlikely to integrate into a homologous single-copy sequence in chromosomal DNA. Instead, foreign DNA might be expected to integrate randomly through a mechanism that uses short sequence homologies, and generates chromosomal deletions.

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Influenza virus RNA is synthesized at fixed sites in the nucleus

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We have recently shown that cellular RNA is synthesized at a sub-nuclear structure, the nuclear cage[1], which contains proteins also found in other structures called variously the nuclear pore complex, lamina, envelope and matrix[2–6]. Is the RNA of an exogenous virus also synthesized at the cage? We chose to study influenza virus as it is unusual in its requirement for a host cell nucleus even though there are no cellular counterparts to the transcription of the infecting negative strands of genomic influenza RNA, nor to the replication of the resulting positive RNA strands to form new virion RNA. The cellular sites of these processes have not yet been definitively demonstrated[7,8]. We now show that nascent viral transcripts are closely associated with the cage and we conclude not only that transcription and replication of viral RNA are nuclear, but also that they occur at fixed sites in the nucleus.

Several observations suggest a nuclear involvement during the transcription and replication of influenza virus[7–10]. These include nuclear labelling in autoradiographs, inhibition of viral production by actinomycin D and α-amanitin—both inhibitors of nuclear RNA polymerase II—and the splicing and methylation of viral mRNA. However, attempts to demonstrate directly the site of viral RNA synthesis by pulse-labelling and cell fractionation have yielded variable results[11–13]. We believe that this variability is caused by long pulse-labels ($\geq$30 min) and nucleolytic action during cell fractionation, both of which permit transfer between cell compartments. We have therefore used very short pulses (2.5 min) and a cell fractionation procedure which minimizes nucleolytic degradation.

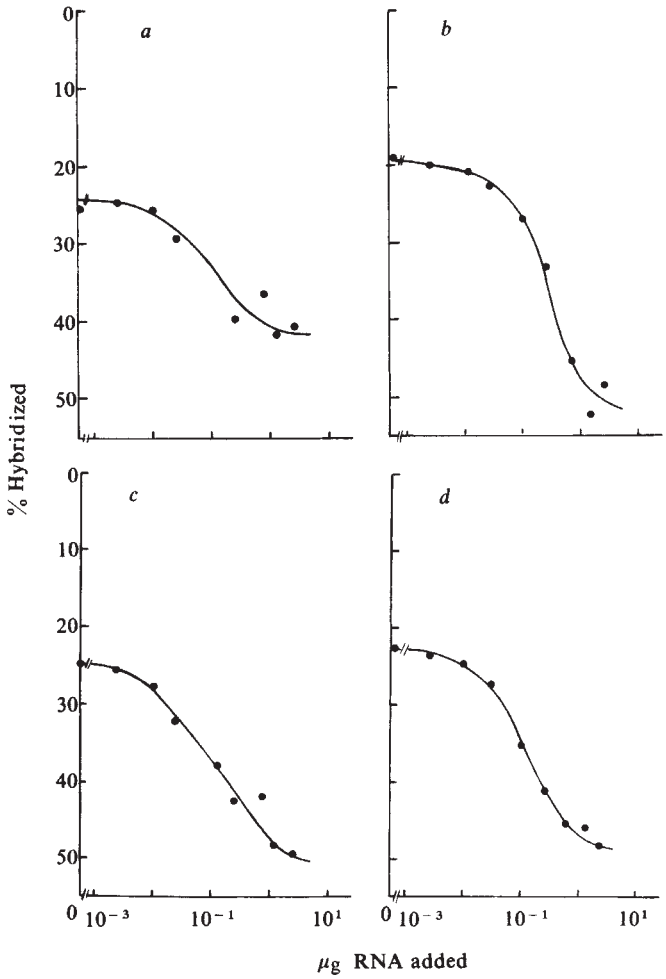

Fig. 1 The proportion of viral transcripts in pulse-labelled RNA from infected cells. Cells were infected and pulse-labelled (2.5 min) with ^{3}H-uridine after 2.5 h (*a*, *b*) or 4.5 h (*c*, *d*). Nucleoids were then isolated as described in Table 1 legend, pulse-labelled RNA was purified and hybridized[1] with various amounts of virus-specific RNA obtained from purified virions (vRNA) or by extracting polyadenylated RNA from the cytoplasm of infected cells 7 h post-infection (mRNA)[18]. (Such a mRNA preparation is impure and contains some cellular RNA, and perhaps low levels of vRNA. However, the cellular impurities have no complements and so cannot form hybrids.) Less than 5% of pulse-labelled RNA from uninfected cells hybridized with these virus-specific RNAs. The per cent forming a hybrid resistant to S_1 nuclease was determined[1,31]. Hybridizations (10 days) were carried out in a volume of 5 μl containing at least 5,000 c.p.m. of pulse-labelled RNA (~2.5 μg of total nucleoid RNA), 2.5 μg tRNA and up to 2.5 μg mRNA (*a*, *c*) or vRNA (*b*, *d*). These conditions give a maximum C_0t of 10^3 mol s l^{-1}.

Embryonic chick fibroblasts were lysed in a non-ionic detergent, 2 M salt and a chelating agent. The resulting nucleoids were sedimented free of cytoplasmic material and contained naked histone-free DNA packaged within a flexible cage of RNA and protein[14–17]. Their DNA, which is looped by attachment to the cage, is supercoiled[14,15], indicating that nucleolytic degradation has been suppressed during isolation.

When mock-infected fibroblasts are incubated with ^{3}H-uridine for 2.5 min, 96% of the cellular radioactivity which is insoluble in trichloroacetic acid subsequently co-sediments with nucleoids (Table 1). Nearly all the label also co-sediments with nucleoids made from infected fibroblasts pulsed at early (2.5 h) or late (4.5 h) times during infection. We determined what proportion of nascent RNA in infected cells was virally coded

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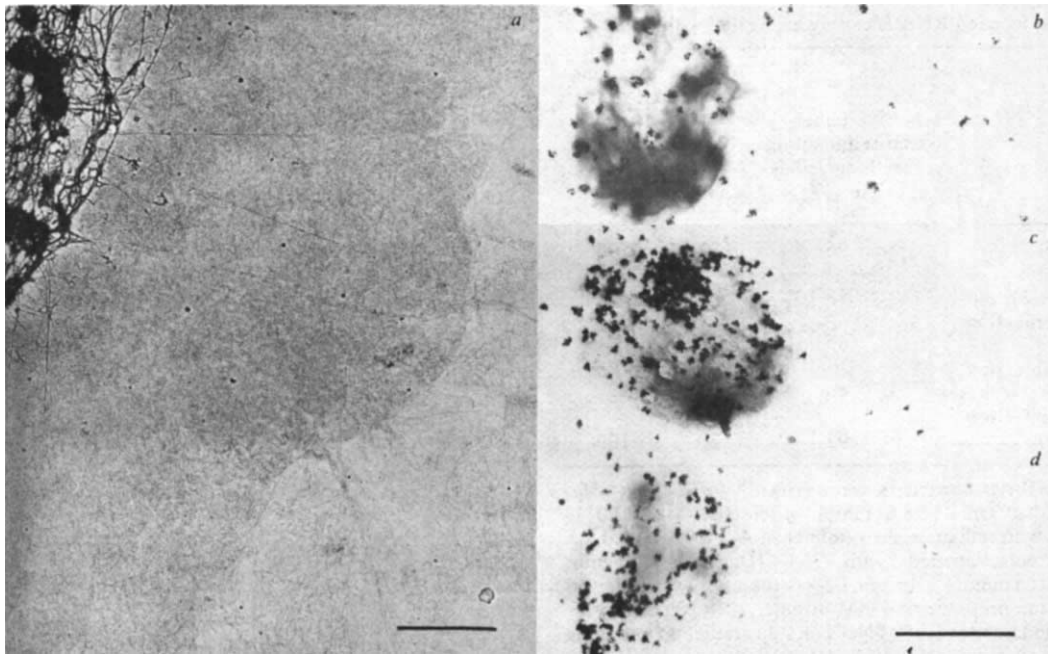

Fig. 2 Electron micrographs of nucleoids spread by Kleinschmidt's procedure. *a*, A typical spread illustrating part of the cage and skirt of tangled DNA fibres, which extend to the edge of the field. *b–d*, Autoradiographs of spreads[19] after labelling uninfected fibroblasts for *b*, 24 h with ^{3}H-thymidine (0.01 μCi ml^{-1}; 58 Ci mmol^{-1}) or for *c*, 2.5 min with [5-6-^{3}H]uridine and (2,5′-8-^{3}H]adenosine (both at 100 μCi ml^{-1} and ~40 Ci mmol^{-1}); or *d*, infected fibroblasts 4.5 h post infection for 2.5 min with ^{3}H-uridine and ^{3}H-adenosine as in *c*. So that silver grains can be easily counted in *b–d*, DNA is shadowed but not stained and can be seen spread to the edge of the field only at higher magnifications. In *b*, grains lie over both cage and skirt, whereas in *c* and *d* they lie predominantly over the cage. Conditions for infection are described in Table 1 legend. The bars in *a* and *d* represent 5 and 2.5 μm respectively. *b–d* Are at the same magnification.

by isolating pulse-labelled nucleoid RNA and hybridizing it with an excess of unlabelled virion (v) RNA or mRNA (Fig. 1), which hybridize with strands of positive and negative polarity respectively[18]. In the absence of added vRNA or mRNA, <1% of the pulse-labelled RNA from mock-infected cells self-anneals, reflecting the asymmetric transcription of cellular DNA. In contrast, about 25% from infected cells self-anneals, reflecting the synthesis of both positive and negative strands. In the presence of an excess of either vRNA or mRNA, 45–50% of the pulse-labelled RNA hybridizes, whereas ~70% hybridizes in the presence of an excess of both vRNA and mRNA (results not shown). We cannot give a precise estimate of the proportion of nascent transcripts which are viral, as our mRNA probe is impure (see Fig. 1 legend) and the significance of the self-annealed values is difficult to assess. Nonetheless, it is clear that positive and negative viral transcripts constitute a significant proportion (>50%) of the pulse-labelled material.

The pulse-label might co-sediment with cages, either because it is specifically attached or because it is tangled in the high concentration of nuclear DNA. We tested the latter possibility by finding out whether RNA could be released on detaching DNA from the cages. Cells were labelled with ^{14}C-thymidine for 24 h to label their DNA uniformly, then infected and subsequently pulsed with ^{3}H-uridine. Nucleoids were isolated, incubated with the restriction endonuclease, *Eco*RI, the cages were filtered free of detached DNA and the amounts of the two labels remaining associated with them determined[19] (Table 1). When >75% of the ^{14}C (that is, DNA) was detached, <1% of the ^{3}H (RNA) was lost, showing that the pulse-labelled RNA cannot be detached with DNA from the cages.

A second experiment confirms that nascent RNA is not simply entangled in DNA or the cage. When nucleoids are spread on an air–aqueous interface, their DNA, initially confined within the cage, spreads out from the cage to form a surrounding skirt of tangled DNA fibres[17] (Fig. 2*a*). Cages from chick fibroblasts are not as robust as those of the HeLa nucleoids that we have studied extensively, so the DNA is less protected from shear. As a result, more is broken and therefore appears relaxed after spreading. The spread DNA is less dense, reflecting the lower DNA content of the diploid chick nucleus. The distribution of DNA in skirt and cage was obtained by reference to autoradiographs of spreads prepared from cells containing uniformly labelled DNA (Fig. 2*b*); 53% (average of 15 spreads) of the DNA is outside the cage. However, when cells—whether infected or not—are pulse-labelled with ^{3}H-uridine for 2.5 min, >85% of the grains lie over the cage in each of 10 spreads selected at random (Fig. 2*c*, *d*). The proportion of grains lying over the nucleolus is reduced in spreads from infected cells (see Fig. 2*c*, *d*) late in infection, reflecting the reduced percentage of ribosomal (cellular) RNA synthesis in the total. The nascent RNA—largely viral—is unable to escape with the DNA from the cage.

A trivial explanation consistent with these results is that nascent viral RNA, synthesized throughout the nucleus, might stick nonspecifically when nucleoids are prepared. Our earlier study[1] showed that nascent RNA in uninfected HeLa cells was specifically attached at the 5′ end, whereas added RNA, or RNA synthesized *in vitro*, was either unattached or was nonspecifically associated with cages but could be detached from them by *Eco*RI or spreading. Using identical conditions we have shown here that nascent viral RNA is inseparable from cages. Three further controls make a nonspecific association even less likely. First, when pulse-labelled RNA from infected cells is purified and mixed with unlabelled infected cells immediately before lysis, <3% of the pure RNA co-sediments with the nucleoids, indicating that the added RNA has little affinity for cages. Second, we have confirmed earlier results[9] which showed by autoradiography that all the pulse-labelled RNA in infected cells was nuclear. After a 2-h chase, the total number of grains over the cell was reduced, reflecting turnover: half lay over the nucleus, the remainder being cytoplasmic (results, not shown, of experiments using conditions described in Table 1). When these cells are lysed in 2 M salt, only 40% of the label now co-sediments with the nucleoids (Table 1). Although

Table 1 Pulse-labelled RNA is closely associated with cages

	% Label co-sedimenting with nucleoids	% Label remaining associated with cages after incubation with *Eco*RI	
	^3H	^{14}C	^3H
Mock-infected cells (2.5 min pulse)	96	17	100
Mock-infected cells (2.5 min pulse, 2 h chase)	58	—	—
Cells 2.5 h post-infection (2.5 min pulse)	93	25	99
Cells 2.5 h post-infection (2.5 min pulse, 2 h chase)	40	—	—
Cells 4.5 h post-infection (2.5 min pulse)	92	20	100

Primary chick embryo fibroblasts were grown[30] for 24 h in [*Me*-^{14}C]thymidine (0.01 μCi ml^{-1}; 58 Ci mmol^{-1}), infected (MOI 100:1) where appropriate with influenza virus (influenza A/PR8/34) and 2.5 or 4.5 h later pulse-labelled with [5,6-^{3}H]uridine (2.5 min; 100 μCi ml^{-1}; ~40 Ci mmol^{-1}). In some cases the pulse was followed by a 2-h chase in the presence of 1 mM uridine. 2.5×10^6 cells were removed, washed and lysed in 1.95 M NaCl on step gradients containing 1.95 M NaCl, spun (Beckman SW50.1 rotor; 15,000 r.p.m.; 45 min) to sediment the nucleoids on to the step and the percentage of ^{3}H co-sedimenting with the nucleoids was determined[15,16]. Nucleoids were collected, diluted to 0.2 M NaCl, incubated with *Eco*RI and the proportion of labels remaining associated with cages was determined after filtration[1,19]. A control experiment showed that >97% of the ^{3}H but none of the ^{14}C could be detached from cages by ribonuclease.

nucleoids contain some elements of the cytoskeleton (that is, actin and intermediate filaments), the cytoplasmic RNA does not co-sediment with the nucleoids.

For the third control we studied Chandipura virus, a rhabdovirus with a single genomic RNA strand of negative polarity and whose reproduction is cytoplasmic[20]. Plaque-purified virus[21] was grown in chick fibroblasts (conditions and multiplicity of infection similar to those described in Table 1 legend so that cytopathic effects were visible after 7 h) in the presence of a concentration of actinomycin D (0.5 μg ml^{-1} added 1 h post-infection) which suppresses nuclear RNA synthesis by >95%. When, at 4 h post-infection, cells were labelled for 10 min with ^{3}H-uridine (50 μCi ml^{-1}), about 3.7 times more label was incorporated into infected cells than into mock-infected controls; thus, 73% of the labelled transcripts were viral, the remainder being the residual host transcripts. When such cells are lysed and spun, only 30% of the incorporated label co-sedimented with nucleoids; 70% was cytoplasmic and remained at the top of the gradient. Unlike nascent influenza virus transcripts, the transcripts of Chandipura virus are not associated with cages.

We have demonstrated that at early and late times during infection with influenza virus, at least 50% of the nascent transcripts in infected cells are viral and all are attached to the nuclear cage. They remain attached in the detergent and 2 M NaCl used to prepare nucleoids. We might have expected little host involvement in RNA-dependent RNA synthesis, especially as the infecting virus contains a polymerase which is capable of synthesizing its own mRNA *in vitro*[22,23]. It is perhaps not surprising that nascent transcripts of positive polarity are cage associated because they are covalently coupled at their 5′ ends to host-encoded sequences[24,25], which we have shown are themselves attached[1]. Attachment of nascent transcripts of negative polarity, which do not contain any host-encoded sequences, is more surprising. However, a complex picture of processing of nuclear transcripts is now emerging. Transcripts are attached to the nuclear cage as they are generated[1] and subsequently as heterogeneous nuclear RNA[26–28]; later they are attached to the cytoskeleton as cytoplasmic message[29]. (This cytoskeletal attachment is unstable in high salt concentrations.) There are obvious practical advantages in tying a long RNA molecule to a larger structure during such a complex series of events as synthesis, capping, methylation, splicing, polyadenylation and translocation to the cytoplasm. There are additional problems associated with the regulated transcription and replication of the eight influenza viral segments and attachment could provide a structural basis for this. Therefore, attachment may play a central part in these processes and any viral nucleic acid subverting some, or all, of them, must also become attached.

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Cytogenetic location and expression of collagen-like genes in *Drosophila*

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Much of the present interest in vertebrate collagens stems from the important part which these extracellular, structural proteins play in developmental processes and tissue organization as well as from their complex gene structure. So far the only vertebrate collagen genes examined encode the constituent polypeptide (pro α) chains of type I procollagen, that is, the pro α2(I) genes from chicken[1,2] and sheep[3], and the pro α1(I) gene from mouse[4]. Recently, we have isolated several collagen-like genomic DNA clones from *Drosophila melanogaster*[5]. In addition to providing data on the evolutionary history of this gene family, studying *Drosophila* has distinct advantages for cytogenetic localization of genes and for defining the functional roles of individual collagens by the application of genetic techniques. Here we compare the hybridization patterns, cytogenetic localization and expression of two of the *Drosophila* clones, DCg1 and DCg2. Although they are cytogenetically unlinked, they share similar developmental RNA profiles.

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