Erratum

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In the print and the online versions of this article the following two references had not been added to the list of references:

Castiglia, C. L. and Flint, S. J. (1983). Effects of adenovirus infection on rRNA synthesis and maturation in HeLa cells. *Mol. Cell Biol.* **3**, 662-671.

and

Stracker, T. H., Carson, C. T. and Weitzman, M. D. (2002). Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. *Nature* **418**, 348-352.

Also, in the PDF version, the size markers were missing from Fig. 4. The correct figure is shown below.

We apologise for the mistakes.
Research Article

Nucleolar protein upstream binding factor is sequestered into adenovirus DNA replication centres during infection without affecting RNA polymerase I location or ablating rRNA synthesis

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Summary

When human adenovirus infects human cells there is disruption of rRNA biogenesis. This report examines the effect of adenovirus infection on the nucleolar protein, upstream binding factor (UBF) which plays a major role in regulating rRNA synthesis. We determined that early after infection, UBF associates with the replication of viral DNA, preferentially associating with the ends of the linear viral genome, and that addition of anti-UBF serum to in vitro replication assays markedly reduced viral DNA replication. Regions of UBF important to these observations are also established. Interestingly, sequestering the majority of UBF from the nucleolus did not lead to the ablation of rRNA synthesis or the sequestration of RNA pol I. In infected cells the bulk of RNA synthesis was RNA pol I associated and distinct from the location of most of the detectable UBF. We propose that UBF plays a role in viral DNA replication, further strengthening the role of nucleolar antigens in the adenovirus life cycle.

Key words: Adenovirus, Upstream binding factor, Nucleolus, Replication

Introduction

Adenovirus infections lead to reorganisation of host cell proteins within the nucleolus and nucleolus (Russell and Matthews, 2003). Castiglia and Flint (Castiglia and Flint, 1983) reported that adenovirus infection leads to nucleolar dysfunction including disruption of rRNA processing and exit of rRNA from the nucleus to the infected cell cytoplasm. Moreover, detailed examination of the nucleolus during adenovirus infection by electron microscopy reveals significant structural changes (Puvion-Dutilleul and Christensen, 1993). However, how this is accomplished, which viral proteins are involved or indeed what benefits the virus derives from these effects is only just beginning to be understood.

Adenoviruses contain a linear dsDNA genome of approximately 36 kb that is replicated in the nucleus of the host cell (Liu et al., 2003; Shenk, 2001). The adenovirus DNA polymerase catalyses the covalent linkage of 5′ terminal nucleotide dCMP to a serine residue on the viral preterminal protein (preTP). This complex binds to the termini of the linear viral DNA and initiates replication of the DNA using the preTP-dCMP molecule as a primer. This occurs in the presence of cellular factors such as NF-1 and Oct-1, which enhance the rate of initiation by binding specific sequences and encouraging unwinding of the viral origin of replication (de Jong et al., 2003; Liu et al., 2003). Once initiated, elongation occurs adjacent to centres in the nucleus that are rich in the viral ssDNA binding protein called DBP. These replication centres are distinct from the nucleolus and from regions of the nucleus where transcription and splicing of viral mRNA occurs (Pombo et al., 1994). Recently, nucleolar antigen B23 has been shown to enhance viral DNA replication (Okuwaki et al., 2001). Our research has demonstrated that at least three adenovirus proteins are targeted to the nucleolus (Lee et al., 2003; Lee et al., 2004; Matthews, 2001) and at least adenovirus protein V can lead to reorganisation of major nucleolar antigens (Matthews, 2001).

The nucleolus is a non-membrane bound nuclear structure where rDNA transcription and ribosome biogenesis occur (Pederson, 1998; Scheer and Hock, 1999). The nucleolus is divided into three distinct substructures known as the fibrillar centres (FC), dense fibrillar centres (DFC) and the granular components (GC). The FC are rich in RNA polymerase I (pol I) and upstream binding factor (UBF). The DFC surrounds the FC and contains pre-rRNA processing factors such as fibrillarin, B23 and nucleolin. The remainder of the nucleolus comprises the GC region, which is enriched with assembly factors and ribosomal proteins.

The synthesis of rRNA begins with formation of the pre-initiation Complex or PIC, consisting of at least upstream binding factor (UBF) and selectivity factor 1 (SL1) that recruits RNA pol I. Several excellent reviews have covered the role of UBF in the transcription of rRNA in detail (Bell et al., 1988; Grummt, 2003; Jantzen et al., 1990; Moss and Stefanovsky, 2002). UBF is a high-mobility group (HMG)-box protein (Jantzen et al., 1990) that plays a key role in the initiation of rRNA synthesis, presumably by helping to anchor the required
factors to the rDNA promoter. Indeed, UBF stays associated with rDNA throughout the cell cycle (Roussel et al., 1993). Moreover, placing heterologous UBF-binding sequences on human chromosomes outside the normal rDNA locations lead to the sequestration of endogenous UBF followed by SL1 and RNA pol I (Mais et al., 2005). To date, however, no clear consensus sequence has been defined but it is likely UBF recognises structural features on DNA rather than a specific sequence (Copenhaver et al., 1994; Kuhn et al., 1994).

Most relevant to our investigation, however, was the observation that UBF is a key regulator of rRNA synthesis (Klein and Grummt, 1999; Liu et al., 1999; Stefanovsky et al., 2001b; Voit and Grummt, 2001; Voit et al., 1999; Voit et al., 1995). We were interested in the disruption of rRNA biogenesis by adenovirus, which include a reported eventual downturn in rRNA synthesis at very late times in infection. Therefore, the location of a number of nucleolar antigens, including UBF, in infected cells was investigated. We saw that UBF is recruited from the nucleolus into viral replication centres where it enhances viral DNA replication. Surprisingly, sequestration of UBF does not lead to significant ablation of rRNA synthesis and we could not detect significant sequestration of RNA pol I to the same sites as UBF in infected cells.

Results

Adenovirus sequesters UBF into sites adjacent to DBP rich replication centres

Immunofluorescence confirms that in uninfected HeLa cells >95% of detectable UBF is observed within the nucleolus as revealed by phase contrast microscopy (Fig. 1A). By contrast, in adenovirus-infected cells >95% of all the detectable UBF was clearly located outside the nucleolus (Fig. 1B). The location of DBP relative to UBF was also examined since DBP is a well characterised marker for viral DNA replication. The in situ location of UBF and adenovirus DBP in adenovirus-infected cells was shown using a directly labelled sheep antiserum to UBF and an indirectly labelled anti-DBP mouse monoclonal antibody. Fig. 1B shows the development of classic ‘doughnut’ structures rich in DBP and the sequestration of UBF adjacent to DBP-rich centres. A commercially prepared antiserum to UBF also corroborated these observations (UBF H-300, from Santa Cruz Biotechnologies, data not shown).

A theoretical possibility was that adenovirus made a protein that antigenically cross reacts with the anti-UBF serum used here. To eliminate this, adenovirus-infected cells were transfected with an EGFP-tagged version of UBF (Chen and Huang, 2001); the distribution of EGFP-UBF was apparently

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Fig. 1. UBF is localised next to viral replication centres. (A-C) UBF is shown in green (anti-UBF serum), DBP in red and the location of the nucleoli is revealed by phase contrast. (A) The location of UBF in uninfected HeLa cells relative to the nucleoli. (B) The location of UBF in adenovirus-infected cells at 16 hours post-infection is shown relative to viral DBP and the nucleoli. (C) Adenovirus-infected cells were examined at 8 hours post-infection when the replication centres are just beginning to form. (D) The location of preTP (in red) and UBF (in green) was examined in infected cells at 18 hours post-infection. The inset in the final merged image is a close up to illustrate that UBF and preTP staining is proximal but does not overlap in the main. All images are of a single focal plane approximately 0.3 μm depth, bar, 10 μm. (E) A western blot of equal numbers of either uninfected cells or adenovirus-infected cells after 16 hours, probed with anti-UBF antiserum to reveal total quantities of protein. The location of the molecular mass markers is shown on the left.
Adenovirus and upstream binding factor

identical to that shown for endogenous UBF (see Fig. 2).

All these experiments confirmed that UBF is sequestered into sites adjacent to the viral DBP centres with some slight overlap in many instances. In addition, the same effects on UBF localisation by adenovirus infection was confirmed in a number of different human cell lines, namely A549 cells, 293 cells and Hep-G2 cells (data not shown).

A low multiplicity of infection (moi) and early time points were used to show that significant sequestration of UBF occurs once DBP has begun to accumulate in discrete centres at approximately 10 hours post-infection (Fig. 1C). Thus, at this time point UBF could be detected in the nucleolus with some UBF sequestered into the emerging DBP-rich centres, whereas the appearance of only a few more small replication centres resulted in the sequestration of all the detectable UBF (as seen in the top cell of Fig. 1B). At 10 hours post-infection there is no detectable late or intermediate gene expression as revealed by immunofluorescence using anti-hexon, fibre, IX or preVI serum (data not shown). In order to exclude early proteins in viral replication, immunofluorescence was used to examine a number of other genes, including E4 ORF3, E1B 55K, L1 52/55K and E1a, without finding any association with UBF (data not shown). The location of pTP relative to UBF at 18 hours post-infection was also examined, showing that both proteins closely associated, but did not colocalise, with each other (Fig. 1D). Finally Fig. 1E shows that by western blotting, infection by adenovirus does not apparently affect the quantity or quality of UBF1 and 2 in HeLa cells compared to uninfected cells. To ensure similar quantities of total protein were present in the two samples, duplicate gels were stained for total protein content (data not shown). In addition, the western blotting data revealed no new bands in adenovirus-infected cells, supporting the idea that the antibodies to UBF do not, fortuitously, cross react with a viral protein. Two different commercially available antisera confirmed that infection by adenovirus did not affect the size or quantity of the observed bands compared to uninfected cells (UBF H-300 and UBF SER-388 from Santa Cruz Biotechnologies, data not shown).

HMG boxes 1, 2 and 3 are required for UBF to accumulate adjacent to DBP

A series of truncated versions of UBF fused to EGFP helped define the regions of UBF important for the association with
DBP (Fig. 2A). These were firstly transfected into HeLa cells in order to assess their intracellular distribution in uninfected cells, then in a series of transfection/infection studies were used to determine how the truncated mutants behaved in adenovirus-infected cells. The data are summarised in Fig. 2B for uninfected HeLa cells and Fig. 2C for infected HeLa cells. In all cases the images are representative of most of the cells examined (i.e. >90%). The location of B23.1 was used as a marker for the nucleolus in uninfected cells and showed that only amino acids 1-192 are needed to localise UBF to the nucleolus but that amino acids 1-572 are needed to give a punctate pattern similar to full-length UBF in uninfected cells. In adenovirus-infected cells, only amino acids 1-362 were required to give a subcellular distribution similar to that of wild-type UBF. This corresponds to HMG boxes 1-3 and implies that DNA binding motifs within UBF are key to the observed location of UBF in infected cells. Interestingly, clones of UBF shorter than this appeared to at least partially colocalise with DBP-rich centres in infected cells. The recruitment appears specific, since in uninfected cells 1-102UBF is diffusely distributed with exclusion from the nucleoli (Fig. 1B). This raises the possibility that we might have uncoupled recruitment and positioning in this system. The N-terminal region (aa 1-102) might be required for recruitment into or nearby the replication centres. Once there, HMG boxes 1-3 of the protein enable UBF to be loaded into its correct location adjacent to the DBP-rich sites.

In all cases western blotting revealed that the majority of the EGFP fusion proteins expressed were full length (data not shown).

The sites of UBF accumulation in adenovirus-infected cells are closely associated with DNA replication

Having excluded a number of viral proteins as interacting partners for UBF, the location of viral nucleic acids was examined, both by defining areas of nucleic acid accumulation and by looking at sites of synthesis.

The data from the deletion series indicated strongly that UBF sequestration was dependent on DNA binding motifs and that UBF might be binding viral DNA. FISH was used to determine that viral nucleic acids could be found at the sites of UBF accumulation in infected cells (Fig. 3A). Labelling of infected cells with fluorouridine (FU) to detect RNA synthesis indicated that a significant role for UBF in the production and processing of adenovirus mRNA was unlikely, since the major sites of RNA synthesis were separate from UBF in infected cells (Fig. 3B).

However, examining DNA synthesis by BrdU incorporation experiments revealed that UBF is associated with the major sites of DNA replication (Fig. 3C,D). In Fig. 3C the cells were pulsed for 30 minutes with BrdU followed by extensive washing of the cells in normal medium followed by a further 2-hour chase. In this case the labelled viral DNA was mostly distinct and did not colocalise with UBF.

Treatment with HCl is routinely used to denature the labelled dsDNA and expose the incorporated BrdU so that it can be detected by antiserum. When this step is omitted and a short pulse time is used, only newly replicated, single stranded DNA contains BrdU that is detectable using antibodies. Using this approach UBF colocalised with those sites that are regions of newly replicated viral DNA either from elongation or replication initiation (Fig. 3D).
Only selected regions of the adenovirus genome are associated with UBF in chromatin immune precipitation (ChIP) assays

We utilised ChIP assays to determine what regions of the viral genome might be associated with UBF. In this assay, infected cells are treated with low levels of formaldehyde in order to reversibly fix interacting DNA and protein. The DNA is sonicated to give a mix of fragments still linked to interacting proteins. Antiserum precipitates the protein of interest along with the DNA sequences that the protein interacts with, and the cross links are reversed to release the DNA. PCR is then used to amplify potential target regions of DNA in order to determine if the protein of interest has indeed bound and precipitated the target DNA. Using this assay, UBF appeared to enrich sequences such as ORF3 from the E4 region, the promoter for the E1 region and the viral packaging signal, all of which are near the ends of the viral DNA, but not the major late promoter, VA RNA I and fibre gene, which are towards the middle of the linear genome (Fig. 4). In all cases, we could not detect any PCR product in uninfected cells using either serum in a ChIP assay (data not shown). This suggested to us that UBF might be involved in events immediately surrounding DNA replication initiation rather than elongation of the nascent strands. If this is the case then other cellular proteins known to associate near the ends of the viral DNA should also be localised in close proximity to UBF. To test this idea the location of double strand break repair proteins was examined in relation to UBF.

UBF associates with double strand break repair (DSBR) proteins in cells infected with ΔE4 adenoviruses

Adenoviruses with a deletion in the viral E4 gene cluster produce concatenated genomes during replication. This is because one of the E4 gene products disrupt the DSBR complex during a wild-type infection, preventing the DSBR complex from joining the linear ends of the viral DNA. A striking similarity between the juxtaposition of DSBR protein Rad50 and DBP in the report by Stracker et al. (Stracker et al., 2002) and the juxtaposition of UBF and DBP observed in this report implied the two proteins might be colocalised in infected cells. Examining the location of Rad50 and UBF in cells infected with an Ad5 ΔE4 type virus, termed dl366, revealed that Rad50 and DBP are closely associated in more than 90% of cells examined (Fig. 5A) and that Rad50 is degraded and mis-distributed in wild-type adenovirus-infected cells (data not shown). Moreover, there is no apparent association between Rad50 and UBF in uninfected cells (Fig. 5B). However, in dl366-infected cells, there is significant association between RAD50 and UBF in more than 90% of cells examined (Fig. 5C,D).

Over expression of EGFP-UBF in infected cells reduces dNTP incorporation in situ

Expression of EGFP-UBF appeared to retard the appearance and number of replication centres in infected cells, potentially reflecting a dominant-negative effect of EGFP-UBF on viral DNA replication. Fig. 6A shows two infected HeLa cells side by side.
by side. In the upper cell there is significant expression of EGFP-UBF with little detectable incorporation of the labelled dUTP. By contrast, the lower cell shows significant incorporation of labelled dUTP but notably less expression of EGFP-UBF. For comparison the same cells are shown but with the exposure levels raised significantly (Fig. 6B) in order to illustrate that both cells contained active replication centres and different amounts of EGFP-UBF. They also have a similar size and number of replication centres. EGFP1-192UBF expression was used as a negative control since this would recruit the EGFP fusion to the replication centres (Fig. 6C). As can be seen, the expression of this fusion and subsequent concentration of it to the replication centres does not apparently affect the incorporation of labelled nucleotides. In the case of Fig. 6A,C the settings of the confocal microscopes for laser power and detection were kept the same so that the images can be compared.

Antiserum to UBF influences incorporation of nucleotides at the viral origins in in vitro adenovirus DNA replication assays

The in situ DNA replication assay was altered to examine if antiserum to UBF could affect viral DNA replication. Thus, antiserum was added to permeabilised infected cells and the levels of DIG-dUTP incorporated into the viral DNA were subsequently detected. In this assay (as with all the previous in situ assays) labelled dNTP would be incorporated into both replication initiation events as well as into DNA at sites across the genome. Permeabilising the cells with Triton X-100 should allow antibodies access to the nucleus as well as the dNTP mixtures. This was confirmed in situ by using the permeabilisation buffer supplemented with anti-B23 serum (2 μg/ml) on cells grown on coverslips. We found that only 4 minutes treatment was enough to allow the entry of the antibody into the nucleus of the cell where it associates with nucleoli (data not shown).

After extraction of viral DNA the viral DNA was digested with KpnI and the recovered fragments were examined by ethidium bromide staining of the agarose gel in the normal manner. The DNA was then Southern-transferred as recommended by the manufacturer’s protocols for the detection of DIG-labelled PCR products.

Fig. 7A shows that the extracted viral DNA was digested as expected by KpnI, yielding the correct fragment sizes. In addition, ethidium bromide staining of the gels ensured equivalent amounts of total viral DNA was present in each lane. Fig. 7B,C show the levels of DIG-dUTP incorporated into each band. As a positive control antiserum against B23 and adenovirus DBP was used, since both these proteins have been shown to play a role in viral replication (Friefeld et al., 1983; Lichy et al., 1983; Okuwaki et al., 2001). For negative controls the anti-UBF serum was pre-incubated with a mixture of UBF1 and UBF2 expressed in baculovirus, the pre-immune antiserum was added at the same concentration, and finally anti-EGFP monoclonal antibody was also tried (from Santa Cruz). In each case there is significantly more incorporation of DIG label into fragments f and g which are the right and left hand ends of the viral genome, respectively. There is some incorporation of DIG along the whole genome reflecting the fact that in this assay we would expect to detect elongation as well as initiation. However, there is a clear decrease in viral replication, particularly in the f and g fragments, when samples are pre-incubated with UBF antiserum. Transfer of known quantities of the DIG-labelled marker was used to semi-quantitate the effects of adding UBF antiserum, and we estimate that there is at least a fourfold decrease in the labelling of the f and g fragments compared to the pre-immune or anti-EGFP-incubated controls (data not shown). However, very accurate quantification is somewhat problematic as the affinities of the different antibodies for their substrates is unknown.

Sequestering UBF does not affect RNA pol I or RNA synthesis in RNA pol I-rich regions of the nucleus

Since UBF is normally always associated with RNA pol I the location of RNA pol I in infected cells relative to UBF, RNA synthesis and DBP was examined. Short pulses of fluorouridine to reveal the location of the bulk of RNA synthesis in both infected and uninfected cells was used. These experiments showed UBF and the bulk of RNA synthesis were colocalised in uninfected cells but spatially separated in adenovirus-infected cells (Fig. 8A,B). In these two figures the images were taken under the same conditions and microscope settings to ensure comparability. This could also be shown using Br-UTP, introduced into cells with Lipofectamine (data not shown). This approach also showed that RNA pol I and UBF were colocalised in uninfected cells and separated in adenovirus-infected cells.
Adenovirus and upstream binding factor (Fig. 8C, D). Thus UBF, RNA pol I and RNA synthesis are colocalised in uninfected cells, but not in infected cells, where RNA pol I and RNA synthesis are separated from UBF. Combining these results showed that FU labelling in infected cells is colocalised with RNA pol I but distinct from DBP (Fig. 8E). Also, in infected cells, Pol I and FU are localised together away from UBF (Fig. 8F).

Duplicate experiments showed that FU labelling of RNA synthesis at Pol I sites could be completely blocked by actinomycin D. This treatment did not affect the localisation of UBF relative to DBP, nor did treatment of cells with DNA replication blockers hydroxyurea or cytosine arabinoside (data not shown).

Initiation of rRNA synthesis is unaffected by adenovirus infection

Examination of RNA synthesis in situ does not distinguish between initiation and elongation. As an alternative method to establish that the initiation of rRNA synthesis is not affected by adenovirus infection, S1 nuclease protection assay was used to examine the levels of rRNA synthesis initiation. Fig. 9 shows that over the time course used in these experiments, and beyond, there is no significant inhibition of rRNA synthesis initiation. Also shown are the total amounts of rRNA loaded on the gel to illustrate that similar amounts of total RNA were included in the protection assay.

Discussion

We have demonstrated that nucleolar protein UBF plays a role in the life cycle of adenovirus and that in virally infected cells, rRNA synthesis continues and is RNA pol I driven despite the sequestration of the majority of UBF into viral replication centres.

This adds UBF to the growing list of cellular proteins recruited by adenovirus during viral DNA replication, and builds upon earlier research showing that nucleolar antigen B23 enhances viral DNA replication (Okuwaki et al., 2001). UBF has been previously reported to colocalise with coiled bodies in infected cells (Rodrigues et al., 1996). However, that report did not directly examine the location of UBF or RNA pol I in relation to viral antigens, and nucleolar antigens were detected using human serum which, in our experience, can also contain antibodies against adenovirus proteins making interpretation difficult (unpublished observations).

The deletion series indicates that UBF might be recruited into the viral replication centres in a two-step process. Region 1-102 is apparently involved in the sequestration of UBF into DBP-rich centres, something we believe is specific since this protein is diffusely distributed in uninfected cells. The localisation of full-length UBF, however, can only be mimicked by fusions containing at least HMG boxes 1-3, suggesting an affinity for a structure involving viral DNA is responsible for the ultimate location of UBF in infected cells. One possible explanation is that the N-terminal region of UBF is attracted through indirect interactions to the viral replication centres. Once recruited the UBF is then loaded onto the viral DNA via an interaction with HMG boxes 1-3 as a minimum. Potentially, the remainder of the protein then plays a functional role. This is in keeping with the observed location of the deletion mutants and our ability to detect viral nucleic acids plus DNA replication at the same site as UBF. In addition, pulse-chase experiments indicate that replicated DNA moves away from the sites of UBF, suggesting that UBF has a dynamic association with the replication complex. Previous
studies on very similar mouse UBF indicate that a region between aa 449 and 480 is required for nuclear localisation (Maeda et al., 1992). The deletion series used here shows that the dimerisation domain and HMG box-I can localise human UBF to the nucleus and nucleolus in uninfected cells. The examination of mouse UBF was done by tagging deletion proteins with the much larger β-galactosidase (~110 kDa) than in our investigation in which we used EGFP fusion proteins, thus there are potentially significant experimental differences that may explain this discrepancy.

In infected cells, DNA replication initiation is adjacent to the DBP-rich centres and distinct from viral mRNA transcription (Pombo et al., 1994). Since we observe UBF adjacent to DBP we would anticipate that UBF is most likely to be associated with the ends of viral DNA where replication initiates. This is supported by both the ChIP assay and by the observations that DSBR proteins colocalise with UBF in dl366-infected cells. When DSBR proteins are associated with replication centres in dl366-infected cells, they promote concatenation of the viral DNA by joining the ends of the genome. The presence of UBF at these sites suggests that UBF is attracted to a DNA-containing structure formed near the origins of replication by viral and cellular proteins.

To shed more light on a potential role for UBF in viral replication, an in situ viral DNA replication assay was utilised. The in situ assay shows that expression of EGFP-UBF retards incorporation of labelled dUTP whereas expression of EGFP1-192UBF does not. Our interpretation of this is that EGFP-UBF acts as a dominant negative protein inhibiting viral DNA replication. This would also fit with our observations that recruitment and function can be separated.

A viral DNA replication assay also shows that DNA replication can be retarded by addition of sera against UBF or against established replication complex components. This is by comparison to the pre-immune sera or an irrelevant antibody. Moreover, addition of purified UBF1 and 2 to the anti-UBF serum prior to addition to the assay restores the replication activity. The clearest effect is on the incorporation of label into the ends of viral DNA (fragments f and g), there is less of an effect on internal fragments (such as d, e and h). Also we noticed that band b appears to respond quite well to the antiserum and this may be a reflection of the fact that on the genome this fragment is next to the left hand most band, band g.

The anti-UBF serum reduces replication approximately fourfold in this assay. Placing this data in context, cellular Oct-1, a well established component of the adenovirus DNA replication machinery, is estimated to influence replication some three to sixfold in vitro (Pruijn et al., 1986). However, the effects of addition of antiserum to a replication assay is difficult to quantify accurately since we do not know the binding kinetics of the antibodies. What is reassuring is that the effects are similar to those using antibodies against DBP and B23.1 and that the inhibition can be mostly restored by adding purified UBF1 and 2.

The origin of replication and minimal protein requirements

Fig. 8. Adenovirus infection causes UBF to separate from RNA pol I and the major sites of RNA synthesis. (A) UBF (in green) and RNA synthesis (detected by FU labelling for 20 minutes as outlined in the methods and shown in red) in uninfected cells. (B) As A, but in adenovirus-infected cells at 18 hours post-infection. C shows the location of UBF (green) and RNA pol I (red) in uninfected cells, and D shows the same in adenovirus-infected cells at 18 hours post-infection. (E) Adenovirus-infected cells were FU labelled as before and simultaneously probed for RNA synthesis (green), RNA Pol I (red) and viral DBP (blue). (F) As for E, but probed for UBF (green), RNA pol I (red) and FU (blue). All images are of a single focal plane approximately 0.3 µm depth; bar, 10 µm.
for initiation events for adenovirus type 2 have been mapped in fine detail. Since most of the first 50 or so bases are bound by viral or cellular cofactors we anticipate that UBF interacts with a viral DNA/protein structure near or just beyond these regions. Our favoured model is that UBF promotes the formation of a protein/DNA complex that enhances viral DNA replication initiation, but this requires further testing. This is consistent with observations that UBF binds cruciform DNA, introduces tight turns in target DNA, does not have strong sequence-specific recognition and can influence chromatin structure (Chen et al., 2004; Copenhaver et al., 1994; Hu et al., 1998; Putnam et al., 1994; Stefanovsky et al., 2001a).

Given the abundance of UBF within a cell it would be unwise to conclude from our results that UBF is not required for continued rRNA synthesis, since low levels of UBF associated with key regulatory elements of the ribosomal gene repeat within nucleoli may not be detected. Future experiments may settle this issue. Clearly, however, we can conclude that the high levels of UBF observed in the nucleoli of uninfected cells may be dispensable in the short term. UBF has been shown to bind across the rDNA repeat and throughout the cell cycle including metaphase. The likely role of extensive UBF binding is to prevent formation of a repressive chromatin structure over rDNA. The formation of such repressive chromatin may not occur within the timeframe of adenovirus infection especially as cells are not going through mitosis.

What is also distinctive in these experiments is the ability to separate UBF from Pol I. Previous reports have shown that where UBF is recruited to sites outside the nucleolus, Pol I is also recruited to these sites (Chen et al., 2005; Mais et al., 2005). We see no evidence of substantial recruitment of Pol I to the same location as UBF in infected cells. Clearly adenovirus induces a unique alteration to UBF (and possibly to Pol I and other proteins involved in rRNA synthesis) as well as other components of the nucleolus. Altered phosphorylation status of relevant proteins or competition for binding sites may explain the lack of colocalisation of these two proteins. However, to date we have not found evidence for a marked difference in the size or quantity of UBF in infected cells (Fig. 1E). This is in contrast to observed alterations to UBF in poliovirus-infected cells, which apparently directly result in rRNA synthesis shut off (Banerjee et al., 2005).

Castiglia and Flint (Castiglia and Flint, 1983) noted that rRNA synthesis inhibition was modest and did not occur until at least 18 hours post-infection, even under their conditions of a very high multiplicity of infection (over 100), which normally accelerates the infectious cycle. In our experiments UBF sequestration is an early event (about 10 hours post-infection) even at low multiplicities of infection and does not markedly inhibit rRNA synthesis even at 30 hours post-infection. Thus, our data is consistent with these earlier reports and leads us to conclude that any eventual decline in rRNA synthesis initiation may well reflect the eventual general decline of the cell as infection progresses.

Experiments are underway to explore further why rRNA biogenesis is affected by adenovirus infection and to further define the roles of nucleolar antigens including UBF in adenovirus infection.

Materials and Methods

Cells and viruses

HeLa cells, cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal calf serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml), were used to propagate human wild-type 2 adenovirus (Ad2). The E4 deletion mutant Ad2 δd66 was kindly provided by Keith Leppard (University of Warwick, UK). Cells were infected with virus at a multiplicity of infection (m.o.i.) of 10 as previously described (Matthews and Russell, 1994). For transfection of plasmid constructs, HeLa cells were grown on glass coverslips in six-well dishes. The cells were transfected with 2 μg of each plasmid using Lipofectamine 2000 (Invitrogen).

Cloning of recombinant derivatives of UBF

Regions of the open reading frame for UBF protein were amplified from pEGFP-UBF (kind donation from S. Huang) (Chen and Huang, 2001) using oligonucleotide primers and a PCR kit (PFX-Invitrogen). The DNA fragments were cloned into a pEGFP-N2 to express the amino acid sequences produced as C-terminal fusions to enhanced green fluorescence protein (EGFP; Clontech). The sequences of primers used in this work are available on request.

Fluorescent imaging

Eighteen hours after transfection, the cells were fixed using 4% formaldehyde (v/v in phosphate-buffered saline; PBS). Cells were washed in PBS, prior to the coverslips being mounted with Vectashield containing 4’,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Detection of EGFP-tagged proteins was performed using a Leica confocal microscope and a 63× oil immersion lens at the University of Bristol MRC cell imaging facility.

Immunofluorescence

Formaldehyde-fixed cells on coverslips were permeabilised using Triton X-100 (1% v/v in PBS) prior to blocking with foetal calf serum (FCS; 1% w/v in PBS) for 30 minutes at room temperature. The following primary antibodies were used: anti-B23 (kindly provided by B. Valdez) (Perlaky et al., 1997), anti-protein V (Matthews and Russell, 1998), anti-protein VI (Matthews and Russell, 1994), anti-DDB (Russell et al., 1989), anti-hexon, anti-penton base (kind donations from W. C. Russell), anti-terminal protein (Webster et al., 1997), anti-UBF (Santa Cruz), sheep anti-UBF, affinity purified and direct labelled with FITC, and sheep anti-rRNA pol I affinity purified and direct labelled with TRITC (Mais et al., 2005). Appropriate secondary antibodies were linked to appropriate Alexa Fluor dyes (Molecular Probes) or to AMCA (Vector Laboratories). Cells were mounted and viewed as described above.

Fluorouridine labelling of transfected cells

HeLa cells were infected with adenovirus as described and 18 hours post-
transfection the cells were incubated with 15 mM 5'-fluorouridine (5'FU, from Sigma) for 15 minutes. Cells were then fixed and permeabilised as outlined and the FISH was performed using commercial anti-BrdU antibody (Sigma) as described previously (Boisvert et al., 2000). Alternatively, the cells were transfected with Br-UTP using Lipofectamine and allowed to incorporate Br-UTP for 1 hour before fixing and detecting incorporated labelled UTP as for FUT.

Fluorescent in situ hybridisation to detect adenovirus DNA

The plasmid pFG140 was purchased from Microbix Biosystems Inc. and contains most of the adenovirus genome. The plasmid was digested with the enzyme Rta to generate a mixture of small fragments that were then random prime labelled using the DIG labelling kit (Roche) as per the manufacturer’s instructions. Immunofluorescence patterns were observed at 18, 26, or 30 hours following infection.

Western blotting

HeLa cells were grown in six-well dishes without coverslips and transfected as described above. After 18-20 hours, cells were harvested and prepared for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was performed using rabbit antibodies against EGF (Santa Cruz), UBF (Santa Cruz) and secondary anti-rabbit immunoglobulin linked to HRP (Vector Laboratories). Detection was performed using ECL (Amersham-Pharmacia).

Infection-transfection studies

To assess the location of EGF-UBF during infection, cells were infected with Ad2 at a MOI of 10 per HeLa cell. This inoculation was performed at the same time as transfection. Although this is not a synchronous infection, more than 90% of cells are infected within an hour of the virus being added. Immunofluorescence patterns were observed at 18, 26, or 30 hours following infection.

ChIP assays

Adenovirus and mock-infected HeLa cells were harvested at 18 hours post-infection and processed for ChIP using a commercially available kit as per the manufacturer’s instructions (Upstate Biotechnology) with just one modification to the protocol. Half of the Sepharose-protein A-linked slurry was substituted for Sepharose-protein G, since protein G is more efficient at binding sheep antibodies. We used both anti-UBF serum and the matched pre-immune serum in our assays. The precipitated DNA was analysed by PCR amplification of selected regions of the viral genome (primers available on request).

S1 nuclease protection assays

10% of the RNA extracted from each plate was electrophoresed on a 1% agarose gel in 1× TAE buffer to assess both the integrity of the RNA sample and the equivalence in RNA concentration between samples. The remainder of each sample was subject to an S1 nuclease protection assay essentially as described previously (Labhart and Reeder, 1986). The probe used was an oligonucleotide 5’- CAACCTCCTCAAGCAAGCCCAGGACCACCGGCTGTCAGAAATACC-CGGGGGGCGCA-3’ labelled on its 5’ end with (γ-32P)ATP and T4 polynucleotide kinase. This probe protects the 5’-42 nucleotides of the 47S pre-RNA. Products were electrophoresed on 8% polyacrylamide, 7 M urea gels in 1× TBE buffer and visualised using a PhosphorImager (Bio-Rad).

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