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A ubiquitin conjugating enzyme encoded by African swine fever virus

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The post-translational modification of proteins by covalent attachment of ubiquitin occurs in all eukaryotes by a multi-step process. A family of E2 or ubiquitin conjugating (UBC) enzymes catalyse one step of this process and these have been implicated in several diverse regulatory functions. We report here the sequence of a gene encoded by African swine fever virus (ASFV) which has high homology with UBC enzymes. This ASFV encoded enzyme has UBC activity when expressed in *Escherichia coli* since it forms thiolester bonds with [^{125}I]ubiquitin in the presence of purified ubiquitin activating enzyme (E1) and ATP, and subsequently transfers [^{125}I]ubiquitin to specific protein substrates. These substrates include histones, ubiquitin and the UBC enzyme itself. The ASFV encoded UBC enzyme is similar in structure and enzyme activity to the yeast ubiquitin conjugating enzymes UBC2 and UBC3. This is the first report of a virus encoding a functionally active UBC enzyme and provides an example of the exploitation of host regulatory mechanisms by viruses.

Key words: ASFV/African swine fever virus/E2 enzymes/UBC enzymes/ubiquitin

Introduction

African swine fever (ASF) is a haemorrhagic fever which affects both domestic and wild swine causing significant economic losses in countries within Europe and Africa where the disease is endemic (Wilkinson, 1990). The causative agent of ASF is a large cytoplasmically located virus particle which replicates in cells of the monocyte/macrophage lineage. It is icosahedral in morphology and contains a long, 170-190 kb, depending on the virus isolate, linear double stranded DNA genome (Vinuela, 1985; Costa, 1990; Dixon *et al.*, 1990a). ASFV shares some important properties with the poxviridae although it is morphologically distinct from them and is classified in a separate virus group of which it is the only member (Brown, 1986). ASFV transcription is not dependent on host cell RNA polymerase and virus particles contain many enzymes, including those required for mRNA transcription and processing (Kuznar *et al.*, 1980; Salas *et al.*, 1981, 1983). Expression of ASFV genes is temporally regulated during infection. Early genes are expressed before replication of virus DNA which, by an unknown mechanism, is necessary for expression of the late genes. In addition, some genes are expressed throughout infection (Costa, 1990).

Ubiquitin is a highly conserved 76 amino acid polypeptide found universally and abundantly in eukaryotic cells. By means of a specific ubiquitin conjugation pathway, ubiquitin is covalently attached to proteins. It has been demonstrated that these ubiquitin-protein conjugates are targeted for proteolytic degradation, however, the presence of metabolically stable ubiquitin conjugates suggests alternative non-proteolytic roles for ubiquitin (Hershko, 1988). The ubiquitin conjugation pathway is a multi-step process. During an initial ATP-dependent activation step, the C-terminus of ubiquitin is attached via a thiolester bond to an internal cysteine of a ubiquitin activating or E1 enzyme. Activated ubiquitin is then transferred to a specific cysteine residue of ubiquitin conjugating (UBC) or E2 enzymes. In the final step ubiquitin is covalently attached to lysine residues of protein substrates. In a subset of reactions this final step requires additional ubiquitin-protein ligases (E3s) (Hershko, 1988; Guarino, 1990; Jentsch *et al.*, 1990). A number of different UBC enzymes have been characterized and these share a relatively conserved N-terminal region of 150 amino acids but have variable length C-terminal extensions (Jentsch *et al.*, 1990). In *Saccharomyces cerevisiae* two UBC enzymes, UBC2 (Jentsch *et al.*, 1987) and UBC3 (Goebl *et al.*, 1988), have very acidic C-terminal extensions and represent the proteins encoded by the previously characterized genes *RAD6* and *CDC34*. *RAD6* mutants are defective in DNA repair and diploid homozygous mutants are deficient in sporulation (Haynes and Kunz, 1981; Lawrence, 1982). The *CDC34* gene is involved in the transition from G_1 to S phase of the cell cycle.

A connection between the ubiquitin conjugation system and virus infection has been suggested by several observations which are not yet understood. A number of plant viruses have been shown to contain ubiquitin-protein conjugates (Hazelwood and Zaitlin, 1990), which have been identified in tobacco mosaic virus as coat protein subunits (Dunigan *et al.*, 1988). The host's ubiquitin conjugating system is stress inducible, therefore these coat protein-ubiquitin conjugates may be formed as part of the host's stress response to the infection. It has also been shown that significant levels of unconjugated ubiquitin are present in avian leukosis virus particles (Putterman *et al.*, 1990), and observations suggest that this results from specific packaging of ubiquitin in virions rather than a spurious event. Free ubiquitin might be involved in virus particle assembly in a similar way to that seen during ribosome biogenesis (Finley *et al.*, 1989). Finally, ubiquitin-like genes have been discovered in the genomes of a togavirus (Meyers *et al.*, 1991) and a baculovirus (Guarino, 1990). A possible role for the products of such genes would be to inactivate a host ubiquitin-dependent cytoprotective system by acting as ubiquitin analogues (Jentsch *et al.*, 1991).

We report here the sequence of a UBC enzyme encoded

by ASF virus and demonstrate that it is functional when expressed in *Escherichia coli*. Its deduced primary structure and its *in vitro* enzyme activity are similar to the yeast UBC2 and UBC3 enzymes. The acquisition by ASFV of a key regulatory enzyme in the ubiquitin conjugating pathway implies that the virus is actively exploiting the host ubiquitin system to its own advantage.

Results

Sequence analysis of a UBC gene encoded by ASFV

We have sequenced part of the genome of a virulent ASFV isolate (Malawi LIL20/1) and compared the amino acid sequences of encoded open reading frames with protein sequence databases. One open reading frame, k121, on the *Sal*Ik fragment (Figure 1) encodes a protein of 213 amino acids with predicted molecular weight of 24 kDa which shares significant homology with a family of UBC enzymes. This open reading frame is located ~15 kb from the right hand DNA terminus and is transcribed towards the centre of the genome (Figure 1). The UBC gene is separated from flanking open reading frames by an upstream AT rich sequence of 158 bp and a downstream region of 622 bp. The upstream region presumably contains the gene promoter. ASFV gene promoters have not yet been defined, although some of the ASFV promoters may be similar to those of vaccinia virus (Hammond and Dixon, 1991). An array of tandemly repeated sequences is located within the 622 bp

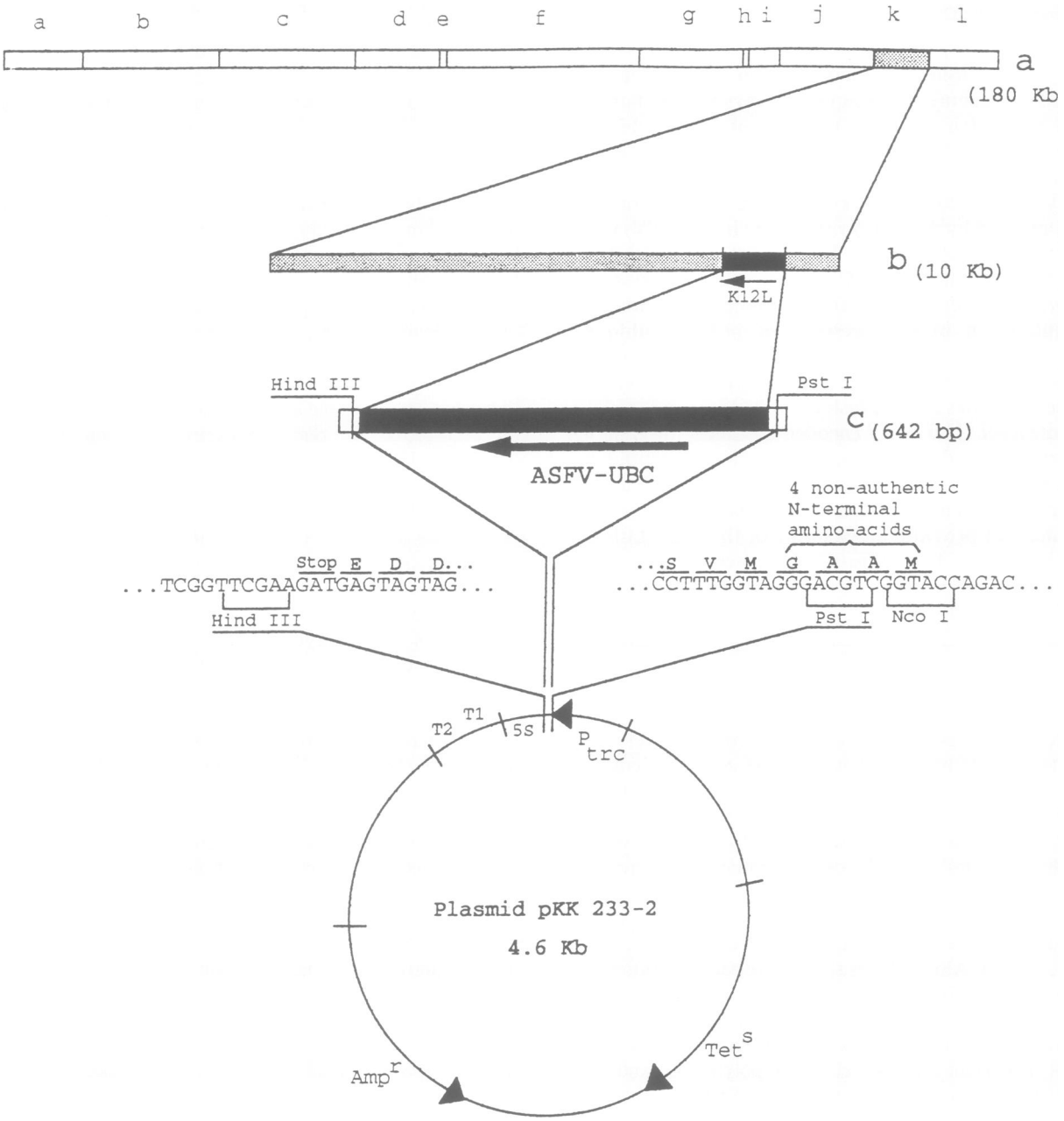

Fig. 1. Genome location and subcloning of ASFV UBC gene.(**a**) shows the *Sal*I restriction enzyme site map of the Malawi LIL20/1 ASFV isolate (Dixon, 1988). The location within *Sal*Ik fragment of the open reading frame which encodes the ASFV UBC enzyme is shown in (**b**). The coding region of this enzyme was amplified by PCR, using primers which included *Pst*I and *Hin*dIII restriction enzyme sites to enable the fragment to be cloned in expression vector pKK233-2 (Amann and Brosius, 1985). This resulted in the addition of four non-authentic codons at the 5′ end of the gene.

downstream region (Figure 2). This is similar in structure to a previously characterized repeat array on the ASFV genome (Dixon *et al.*, 1990b), although the sequences of current repeat units diverge for those published previously.

The ASFV encoded UBC enzyme has between 31 and 45% identical amino acids in the conserved N-terminal region when compared with other UBC enzymes. Similarity between the ASFV and other UBC enzymes is between 52 and 66% when conservative amino acid substitutions are included. Alignment of the ASFV UBC enzyme sequence with that of the yeast UBC2 and UBC3 enzymes, with which the highest percentage homologies were observed, enables the most conserved amino acids to be identified (Figure 3). The amino acid sequence surrounding the active site cysteine in the ASFV UBC enzyme is most closely related to that of the yeast UBC3 gene (Figure 3), since the residues are conserved and both genes contain extra amino acids downstream from the active site which are not present in other UBC enzyme sequences. Other conserved regions include residues close to the C-terminus of the common do-

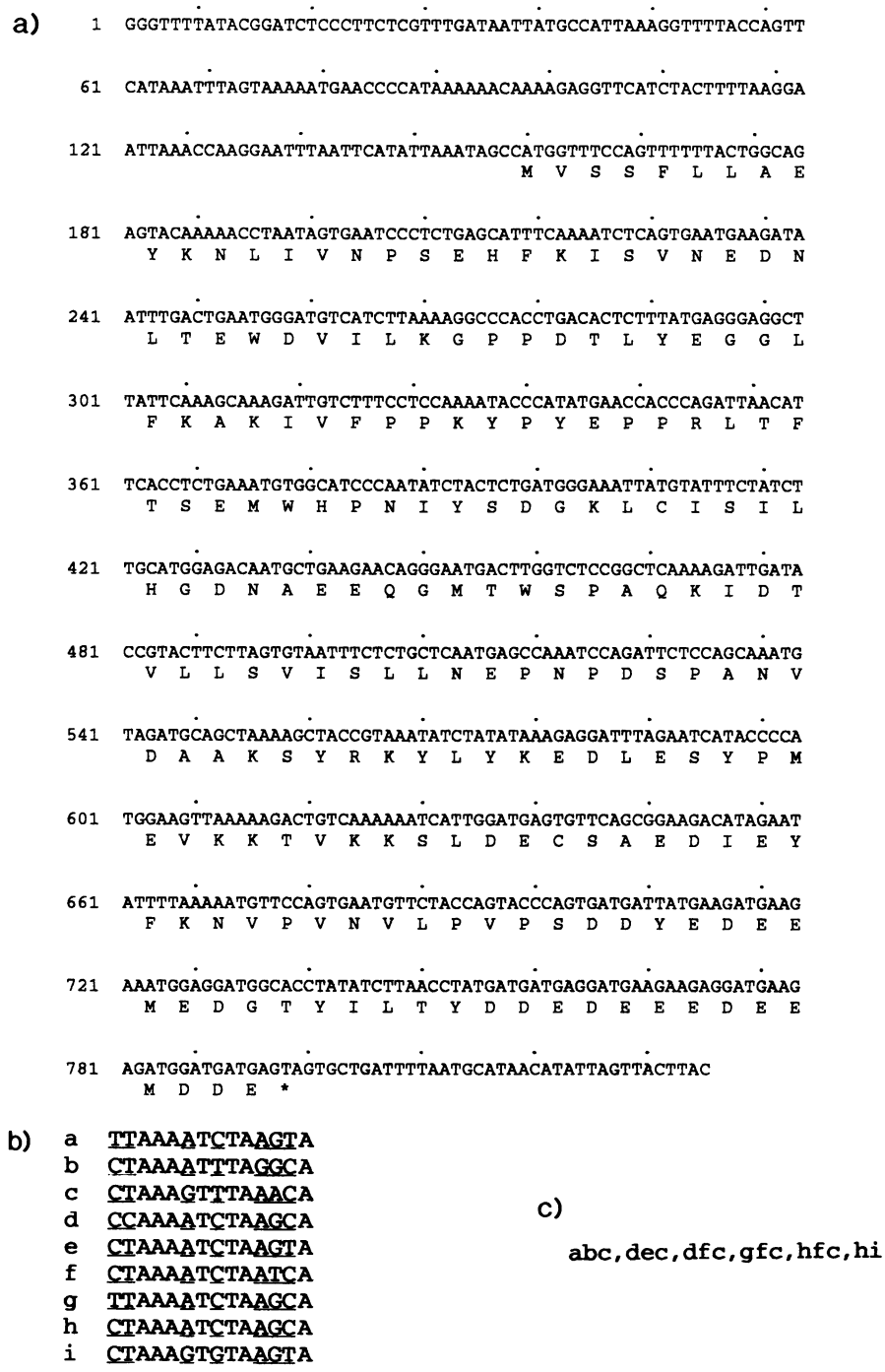

Fig. 2. Nucleotide sequence of the coding region of the ASFV UBC gene and flanking sequences. The nucleotide sequence of the coding strand of the ASFV UBC gene and flanking sequences is shown in (**a**). The amino acid sequence of the UBC gene is shown below the nucleotide sequence. In (**b**), sequences of different types of repeat units in the tandem repeat array are shown. Positions of sequence divergence are underlined. This repeat array is located downstream from the coding region and flanking sequence of the UBC gene shown in (a). Sequences shown are complementary to the UBC gene coding strand. In (**c**), the arrangement of these different types of repeat units within the repeat array is indicated.

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                1                                                          60

ASFV UBC  ......MVSS FLLAEYKNLI ....VNPSEH FKISVNEDNL TEWDVILKGP .PDTLYEGGL
UBC2      ......MSTP ARRRLMRDFK RMKEDAPPGV SASPLP.DNV MVWNAMIIGP .ADTPYEDGT
UBC3      MSSRKSTASS LLLRQYRELT DPKKAIPSFH IELEDD.SNI FTWNIGVMVL AEDSIYHGGF
                    .         ..          :            :.   :.  .      :. :  :

                61                                     *                   120
ASFV UBC  FKAKIVFPPK YPYEPPRLTF TSEMWHPNIY SDGKLCISIL HGDN....AE EQGMTWSPAQ
UBC2      FRLLLEFDEE YPNKPPHVKF LSKMFHPNVY ANGEICLDIL QNR.......  .....WTPTY
UBC3      FKAQMRFPED FPFSPPQFRF TPAIYHPNVY RDGRLCISIL HQSGDPMTDE PDAETWSPVQ
           :.  . :     :  :: . :  . ..::::.:  .: .:. ::                 :.:

               121                                                         180
ASFV UBC  KIDTVLLSVI SLLNEPNPDS PANVDAAKSY RKYLYKEDLE SYPMEVKKTV KKSLDE....
UBC2      DVASILTSIQ SLFNDPNPAS PANVEAATLF KD.....HKS QYVKRVKETV EKSWED....
UBC3      TVESVLISIV SLLEDPNINS PANVDAAVDY RK.....NPE QYKQRVKMEV ERSKQDIPKG
            . ..: :.  :: ..::  : ::::.::    .           :   ::  :  .:  .

               181                                                         240
ASFV UBC  .......... ...CSAEDIE YFKNVPVNV. .......... ..LPVPSDDY ED........
UBC2      .......... .......... .......... .......... .......... ..........
UBC3      FIMPTSESAY ISQSKLDEPE SNKDMADNFW YDSDLDDDEN GSVILQDDDY DDGNNHIPFE


               241                                                         300
ASFV UBC  .......EEM EDGTYILTYD DEDEEEDEEM DDE
UBC2      .......... DMDDMDDDDD DDDDDDDEAD
UBC3      DDDVYNYNDN DDDDERIEFE DDDDDDDDSI DNDSVMDRKQ PHKAEDESED VEDVERVSKKI
                     .        . :.:...:.
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Fig. 3. The deduced amino acid sequence of the ASFV UBC enzyme was aligned with those of the yeast UBC2 (Jentsch *et al.*, 1987) and yeast UBC3 enzymes (Goebl *et al.*, 1988) using the program GAP (Devereux *et al.*, 1984). Sequences near the N-terminus were aligned by eye to fit previously published comparisons of the yeast UBC2 and UBC3 sequences. Residues that are identical in the ASFV and one of the yeast sequences are shown in outline. The active site cysteine is indicated by an asterisk above the sequence. Residues that are identical in all three sequences are underscored with a double dot, those residues where conservative amino acid changes occur are underscored with a single dot.

main. Sequences close to the N-terminus are more divergent. The C-terminal extensions of these enzymes are variable in length but all have a high percentage of acidic residues.

Ubiquitin acceptor capability

The presence of the active site cysteine and other conserved amino acids in the ASFV UBC enzyme sequence indicates that the enzyme may be functional. To confirm this we cloned the ASFV UBC enzyme coding region in the *E.coli* expression vector pKK233-2 (Figure 1) (Amann and Brosius, 1985). In the presence of ATP and affinity-purified E1 from pig brain, [^{125}I]ubiquitin was transferred to a protein which was induced in bacteria containing the expression plasmid with the UBC gene but not in bacteria containing the expression plasmid without the insert (Figure 4a). Transfer of [^{125}I]ubiquitin to this protein was dependent on the presence of both ATP and E1 enzyme. The apparent molecular weight of the [^{125}I]ubiquitin−protein complex on SDS−PAGE was 36 kDa which is the predicted molecular weight of a covalent complex consisting of ubiquitin and the additional 30 kDa protein induced in bacteria containing the expression plasmid. The discrepancy in molecular weight between that predicted from the amino acid sequence and estimated by PAGE is presumably due to aberrant migration of the enzyme on the gels. The [^{125}I]ubiquitin was removed from the UBC enzyme when samples were heat treated in the presence of 2-mercaptoethanol prior to loading on the gel, indicating that [^{125}I]ubiquitin was linked to the UBC enzyme by a thiolester bond (Figure 4a).

Ubiquitin conjugation activity

Having established that *E.coli*-expressed ASFV UBC enzyme could form thiolester bonds with [^{125}I]ubiquitin in a reaction that was dependent on added ATP and E1 we investigated whether the [^{125}I]ubiquitin could be transferred from the *E.coli*-expressed UBC enzyme to a protein substrate. *In vitro* assays showed that the *E.coli*-expressed enzyme was able to transfer [^{125}I]ubiquitin to protein substrates with the formation of covalent isopeptide bonds in E3-independent reactions (Figure 4b). In reactions with no added protein substrate, ubiquitin conjugates with molecular weights of 17 kDa and a ladder of products between 36 and 57 kDa were formed. These are likely to represent di-ubiquitin (Chen and Pickart, 1990) and multi-ubiquitinated conjugates of the UBC enzyme respectively. Two additional ubiquitin conjugates of molecular weights 26 and 32 kDa were formed when histone H2B was added to the reaction (Figure 4b). The molecular weight of these conjguates suggest that they are the multi-ubiquitinated H2B-Ub_2 and H2B-Ub_3 forms of histone H2B.

Discussion

Our results show that the ASFV UBC enzyme can form ubiquitin conjugates in at least three separate ways; these include the capacity to form multi-ubiquitinated conjugates of histones, di-ubiquitin and multi-ubiquitinated UBC enzyme. In common with the ASFV UBC enzyme, other UBC species can transfer ubiquitin to histones in an

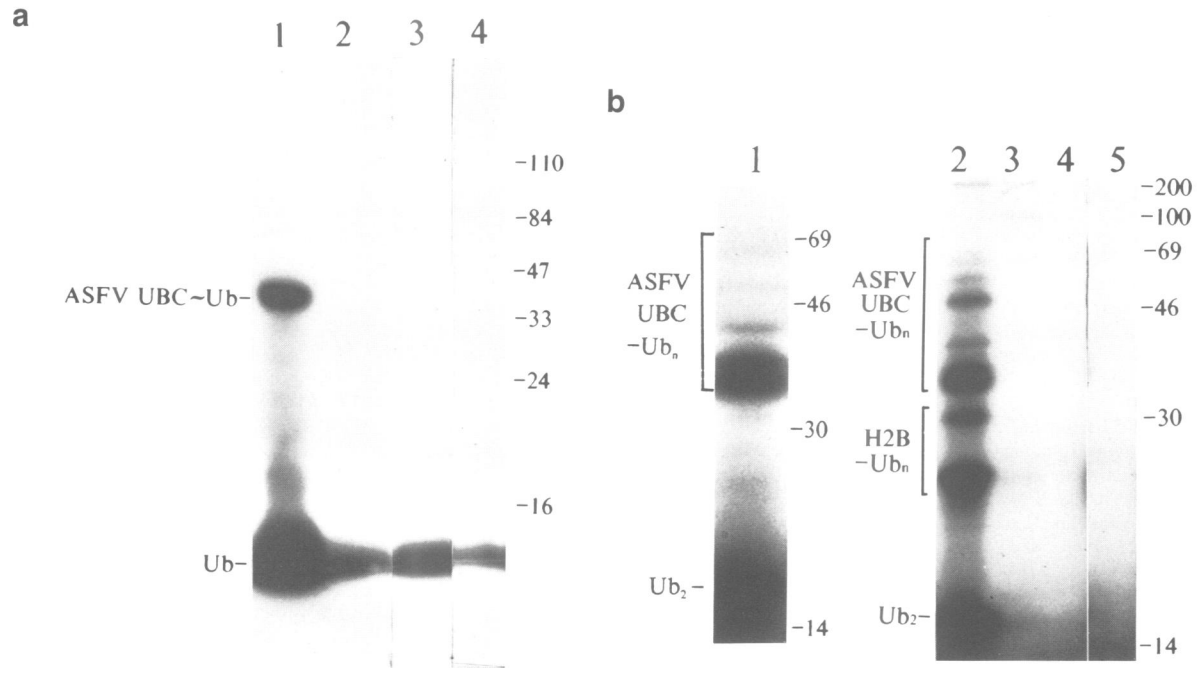

Fig. 4. *In vitro* assays of the *E.coli*-expressed ASFV UBC enzyme (autoradiographs of SDS−PAGE). (**a**) Formation of a thiolester bond between ASFV UBC enzyme and [^{125}I]ubiquitin. Reactions contained *E.coli*-expressed ASFV UBC enzyme, [^{125}I]ubiquitin, E1 enzyme and ATP. In lanes 2 and 3, E1 and ATP respectively were omitted. In lane 4, the sample was submitted to reducing conditions (see Materials and methods) prior to analysis. (**b**) Transfer of [^{125}I]ubiquitin to protein substrates. Reactions contained *E.coli*-expressed ASFV UBC enzyme, [^{125}I]ubiquitin, E1 enzyme and ATP. Histone H2B was included in reactions shown in lanes 2−5. In lanes 3 and 4 either E1 (lane 3) or ATP (lane 4) were omitted. In lane 5, the *E.coli* extract containing the ASFV UBC enzyme was replaced by an equivalent extract from *E.coli* cells containing the plasmid without the insert. The positions at which molecular weight markers ran are indicated in kDa, as are protein species that are referred to in the text.

E3-independent reaction (Jentsch *et al.*, 1987; Goebl *et al.*, 1988; Sullivan and Viestra, 1989; Berleth and Pickart, 1990). The acidic C-terminal extension of the UBC2 and UBC3 enzymes are required for efficient *in vitro* conjugation of ubiquitin to histones (Sung *et al.*, 1988; Goebl *et al.*, 1988) and are therefore probably important for interaction with protein substrates which *in vivo* may include histones or other basic proteins. In the nucleus of higher eukaryotes apparently metabolically stable ubiquitin−histone conjugates are found. Their formation may in some way alter chromosome structure, making DNA accessible for other regulatory proteins. The *in vivo* significance of the *in vitro* histone conjugation activity of the ASFV UBC enzyme is unknown, since it is not yet known whether the enzyme is localized within the cytoplasm or nucleus during virus infection.

Multi-ubiquitination of target proteins is known to occur during protein degradation in the ATP and ubiquitin-dependent proteolytic pathway (Chau *et al.*, 1989), and di-ubiquitin may be an intermediate in forming the branched poly-ubiquitin chains (Chen and Pickart, 1990). The ability of the ASFV UBC enzyme to multi-ubiquitinate substrates may therefore suggest a role in targeting substrates for proteolysis rather than modulating protein substrate function in a non-proteolytic manner. Specific examples of regulatory proteins whose degradation appears to involve ubiquitinations are cyclins (Glotzer *et al.*, 1991), a yeast transcriptional regulator (Hochstrasser *et al.*, 1991) and oncoproteins (Ciechanover *et al.*, 1991). The observed multi-ubiquitination of the ASFV UBC enzyme may serve to regulate its biological activity or intracellular stability.

The acquisition of a UBC enzyme by ASFV enables the virus to exploit the host ubiquitin conjugation system either to regulate the virus replication cycle or to regulate in some other way the virus−host cell interactions. Possible stages of the virus replication cycle in which the virus-encoded UBC enzyme may be involved include uncoating or assembly of virus particles, regulating the transition from early to late gene expression, regulating the onset of virus DNA replication or in virus DNA repair. Alternatively, ASFV may have evolved ubiquitin-dependent mechanisms to modulate monocyte/macrophage function. The similarity in structure of the ASFV UBC enzyme to that of the yeast UBC3 enzyme may indicate that ASFV UBC has a similar cell cycle regulatory function. Such a function might also affect the terminal differentiation of monocytes. The recent findings of a specific enrichment of ubiquitin protein conjugates in lysosmes (reviewed in Mayer *et al.*, 1991) suggests an alternative role for the ASFV UBC enzyme in manipulation of the endosome−lysosome system of monocytes or macrophages to the advantage of the virus.

Materials and methods

DNA sequencing

The *Sal*Ik fragment of the Malawi LIL20/1 genome was isolated from bacteriophage λ clone LMw22 (Dixon, 1988). A library of randomly sheared fragments was cloned in M13 (Bankier *et al.*, 1988) and sequences of inserts were determined by the chain termination method (Bankier *et al.*, 1988). Sequences were assembled into a contiguous fragment by computer (Staden, 1982) and deduced amino acid sequences of encoded open reading frames compared with protein sequence databases using the Fasta program (Lipman and Pearson, 1985).

Cloning of ASFV UBC in expression vector pKK233-2

The ASFV UBC gene was amplified from *Sal*Ik fragment by polymerase chain reaction (PCR) using DNA from bacteriophage λ clone LMw22 (Dixon, 1988) as template. Primers used in the reaction included sequences from the 5′ and 3′ end of the gene and added *Pst*I and *Hin*dIII sites. These were, at the 5′ end ACCGGTCTGCAGGGATGGTTTCCAGTTTTT-

TACT, and at the 3′ end GTGTGAAAGCTTCTACTCATCATC-CATCTCTT. DNA was amplified using 25 cycles of 2 min at 92°C, 2 min at 45°C and 3 min at 72°C. The PCR product was digested with *Pst*I and *Hin*dIII and cloned into *Pst*I, *Hin*dIII cut vector pKK233-2 (Amann and Brosius, 1985).

Purification of ASFV UBC

ASFV UBC was partially purified from *E.coli* by DEAE cellulose chromatography. A 1 l culture of *E.coli* harbouring plasmid pKK233-2 with the insert was grown to mid-exponential phase and expression of the UBC enzyme was induced by adding 1 mM IPTG. After a further 90 min incubation, cells were lysed by freeze–thawing in 50 mM Tris–HCl pH 7.5/1 mM EDTA/0.1 mM DTT. The lysate was centrifuged at 20 000 r.p.m. in a Beckman SW28 rotor and the supernatant applied to a 20 ml DEAE cellulose column. Fraction II was concentrated to 2 ml (Amicon Centriprep-10) and 10 μl were used for UBC activity assays.

Purification of E1 from pig brain

E1 was purified from 30 g of pig brain tissue by ubiquitin–Sepharose affinity chromatography. A pig brain fraction II was obtained by DEAE cellulose chromatography. Fraction II was concentrated (Amicon Centriprep-10) and applied to 2 ml ubiquitin–Sepharose column (5 mg ubiquitin/ml of gel) in the presence of 5 mM ATP/10 mM $MgCl_2$/50 mM Tris–HCl pH 7.5. E1 was specifically eluted with 2 mM AMP/0.04 mM $NaPP_i$ in 50 mM Tris–HCl pH 7.5 (Ciechanover *et al.*, 1982). The eluate was concentrated to 500 μl (Amicon Centriprep-3) and 15 μl were used for activity assays.

UBC enzyme assays

Assays for the formation of a thiolester bond betwen ASFV UBC enzyme and [^{125}I]ubiquitin were carried out by incubating extracts from *E.coli* containing the expressed ASFV UBC enzyme for 15 min at 37°C in reaction mixtures containing 50 mM Tris–HCl pH 7.5/5 mM ATP/5 mM $MgCl_2$ with [^{125}I]ubiquitin (10^6 c.p.m.) and 1 U of inorganic pyrophosphatase in the presence of pig brain E1. Reactions were stopped by adding sample buffer (15 mM Tris–HCl pH 6.8/100 mM DTT/2% SDS/0.1% bromophenol blue/20% glycerol). To test ATP dependence, $MgCl_2$ and ATP were replaced by 5 mM EDTA. Reducing conditions were obtained by boiling the reaction mixture in 5% 2-mercaptoethanol prior to analysis. Products of the reactions were electrophoresed in 20% SDS–PAGE gels and detected by autoradiography. Assays for transfer of [^{125}I]ubiquitin to protein substrates were performed and analysed as described for thiolester assays except that the incubation time was extended to 90 min and 50 μg of histone H2B was added to some reaction mixtures. All conjugation assay samples were boiled in 5% 2-mercaptoethanol prior to electrophoresis.

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