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The 2A proteins of three diverse picornaviruses are related to each other and to the H-rev107 family of proteins involved in the control of cell proliferation

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The 2A protein appears to be diverse among picornaviruses, in contrast to the other non-structural proteins, which have homologous structures and functions. In enteroviruses and rhinoviruses, 2A is a trypsin-like protease involved in protein processing and in shut-off of host-cell macromolecular synthesis. The aphthovirus and cardiovirus 2A is associated with an unusual processing event at the 2A/2B junction. It is shown here that the 2A protein of several diverse picornaviruses, the human parechoviruses, Aichi virus and avian encephalomyelitis virus, possess previously unrecognized conserved motifs and are likely to have a common function. Moreover, these motifs, a conserved histidine and flanking amino acids, an asparagine–cysteine dipeptide and a putative transmembrane domain, are characteristic of a family of cellular proteins, at least two of which are involved in the control of cell growth. These observations have important implications for an understanding of picornavirus genome structure and evolution, as well as pointing to possible functions of 2A in these viruses.

Introduction

The family *Picornaviridae*, which contains several important human and animal pathogens, is a large and diverse family of positive-sense RNA viruses (Stanway, 1990; Rueckert, 1996). More than 230 serotypes have been recognized and are currently grouped into six genera: *Aphthovirus*, *Cardiovirus*, *Enterovirus*, *Hepatovirus*, *Parechovirus* and *Rhinovirus* (King *et al.*, 1999; Stanway & Hyypiä, 1999). Another three viruses, Aichi virus, porcine enterovirus 1 [to be renamed porcine teschovirus 1 (PTV1)] and equine rhinotracheitis virus B (ERBV), are likely to be classified as type members of new genera in the future, as they exhibit significant diversity from members of existing genera (Wutz *et al.*, 1996; Yamashita *et al.*, 1998; Doherty *et al.*, 1999; King *et al.*, 1999).

The picornavirus genome is 7000–9500 nucleotides long and is enclosed within a naked particle, made up of 60 copies of each of the capsid proteins. The genome encodes a single polyprotein, which undergoes a cleavage cascade performed by virus-encoded activities, to give the final virus proteins. The genomes of the various genera encode 10, 11 or 12 final proteins, but intermediates in the cascade can have a significant half-life and may have distinct functions (Rueckert, 1996). All picornaviruses share essentially the same genome organization. The capsid proteins (1A, 1B, 1C and 1D, commonly known as VP4, VP2, VP3 and VP1, respectively) are encoded towards the N terminus of the polyprotein and the non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D) are encoded downstream of these. VP4 and VP2 are assembled into the particle in the form of a precursor, VP0, the final step of assembly being the cleavage of this precursor, which appears to be required for virion infectivity and stability. However, in the human parechoviruses and in Aichi virus, this cleavage appears not to occur and consequently these particles have only three proteins, VP0, VP3 and VP1 (Hyypiä *et al.*, 1992; Stanway *et al.*, 1994; Yamashita *et al.*, 1998).

For most picornavirus proteins, there is clear homology between the corresponding protein-encoding region of different genera. One exception is L, the protein encoded at the extreme N terminus of the polyprotein in aphthoviruses, cardioviruses, ERBV and Aichi virus (Rueckert, 1996; Wutz *et al.*, 1996; Yamashita *et al.*, 1998). With the exception of aphthoviruses and ERBV, where they are homologues, picornavirus L proteins are structurally and functionally distinct (Rueckert, 1996). The other variable picornavirus locus is the

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region encoding 2A. In rhinoviruses and enteroviruses, 2A is a trypsin-like, cysteine protease involved in polyprotein processing, while in cardioviruses, aphthoviruses, ERBV and PTV1, it is associated with an unusual processing activity, involving an Asn–Pro–Gly–Pro (NPGP) motif (Ryan & Flint, 1997). The 2A proteins of parechoviruses, hepatoviruses and Aichi virus are not believed to be involved in processing (Schultheiss *et al.*, 1995; Jia *et al.*, 1993; Yamashita *et al.*, 1998). In addition, no similarity between the 2A protein of parechoviruses, hepatoviruses and Aichi virus has previously been noted.

We show here that the 2A proteins of parechoviruses are, in fact, homologous to the corresponding region of the polyprotein of the hepato-like virus, avian encephalomyelitis virus (AEV), and, to a lesser extent, to the 2A protein of Aichi virus (Marvil *et al.*, 1999; Yamashita *et al.*, 1998). Moreover, these proteins are also related to a recently identified family of cellular proteins, possibly involved in the control of cell proliferation. These observations have important implications for an understanding of the replication of these viruses and of picornavirus evolution.

Methods

■ **Sequences.** The sequences used in this work are in the EMBL database, under the following accession numbers: human parechovirus 1 (HPeV1), strain Harris (L02971); human parechovirus 2 (HPeV2), strain Williamson (AJ005695); human parechovirus 2, strain 86-6760 (Connecticut/86) (AF055846); AEV, Calnek vaccine strain (AJ225173); Aichi virus, strain A846/88 (AB010145); human H-rev107 (X92814); rat H-rev107 (X76453); lecithin retinol acyltransferase (LRAT) (AF071510); Tazarotene-induced gene protein (TIG3) (AF060228); Southampton virus (L07418); Norwalk virus (M87661); and Lordsdale virus (X86557).

■ **Sequence comparisons.** Database searches were performed by using the EBI FASTA3 (http://www2.ebi.ac.uk/fasta3/) or BLAST2 (http://www2.ebi.ac.uk/blast2/) search facilities (Pearson & Lipman, 1988; Altschul *et al.*, 1990). A range of FASTA3 parameters was used to analyse the HPeV1 sequence and a match with AEV was found by using the blosum50 matrix, Ktup = 2, GapPenOpen = −12 and GapPenExtend = −2. These parameters were used for all other searches. Clustal W (http://www2.ebi.ac.uk/clustalw/) was used to align multiple sequences (Thompson *et al.*, 1994).

■ **Sequence analysis.** Sequences were analysed for the presence of likely transmembrane domains by using the program TMpred (http://www.ch.embnet.org/software/TMPRED form.html; Hofmann & Stoffel, 1993). The program ppsearch (http://www2.ebi.ac.uk/ppsearch/) was used to perform PROSITE pattern searches.

Results and Discussion

It has been shown previously that HPeV1 (originally known as echovirus 22) is a typical picornavirus, but has a low degree of identity to most other members of this family (Hyypiä *et al.*, 1992; Stanway *et al.*, 1994). Consequently, HPeV1 has been assigned as the prototype of a new genus, *Parechovirus*, which also includes the closely related HPeV2, two strains of which have now been sequenced (Ghazi *et al.*, 1998; Oberste *et al.*, 1998; King *et al.*, 1999; Stanway & Hyypiä, 1999). The 2A protein of parechoviruses was believed to lack homology to the corresponding proteins from other picornaviruses and its function is not clear. Previous attempts to identify related proteins, using FASTA3 or BLAST searches of protein databases, in order to gain insights into possible functions, yielded no significant matches (Hyypiä *et al.*, 1992; Ghazi *et al.*, 1998). More extensive comparisons were therefore undertaken, employing a range of parameters to search the SWISS-PROT database with the HPeV1 2A sequence as the search string. These revealed a significant match with the corresponding region of the polyprotein of the recently sequenced picornavirus AEV (Marvil *et al.*, 1999) (Fig. 1). The published sequence of AEV predicts that, in this virus, 2A is short and therefore the observed region of homology extends into the predicted 2B-encoding region. However, it is clear that the genomic position, immediately downstream of the capsid protein-encoding region, is the same in both cases. The protein sequences are relatively diverse, but there are two regions of clear similarity. One is centred on amino acid 24 (His) of the parechovirus 2A and the other is a Lys–Asn–Cys–Glu–Thr (KNCET) motif (positions 91–95 in HPeV1 2A). Moreover, the latter motif is almost equidistant from a long (18 amino acids) hydrophobic domain in the AEV and HPeV1 proteins.

In view of these similarities, FASTA3 and BLAST database searches were performed, with the homologous region from AEV as the search string. These revealed a significant match with a human protein, H-rev107, and its rat homologue (Hajnal *et al.*, 1994; Sers *et al.*, 1997; Husmann *et al.*, 1998). Further searches with H-rev107 as the search string confirmed an already observed similarity to the Tazarotene-induced gene protein 3 (TIG3), also called retinoic acid-responder 3 protein (DiSepio *et al.*, 1998), and identified a previously unreported match with the enzyme lecithin retinol acyltransferase (LRAT) (Ruiz *et al.*, 1999). All these cellular proteins have a long, hydrophobic domain and an Asn–Cys–Glu (NCE) motif resembling the conserved KNCET motif of HPeV1 and AEV. In addition, they exhibit similarity to the virus proteins in the HPeV1 position 24 region. Finally, inspection of the 2A proteins of other picornaviruses indicated that the recently described Aichi virus, which constitutes a distinct genetic lineage among picornaviruses, also has these features, in a weaker form, but that they are absent from other members of this family, including hepatitis A virus, the closest relative of AEV (Yamashita *et al.*, 1998; Marvil *et al.*, 1999).

It can be seen from the alignment of the homologous region of the three available HPeV sequences, AEV and Aichi virus, together with the cellular proteins, that there is conservation of all the sequence features described above (Fig. 1). In addition, the HPeV1/2 and Aichi 2A proteins are virtually co-N-terminal with H-rev107 and TIG3 and have a

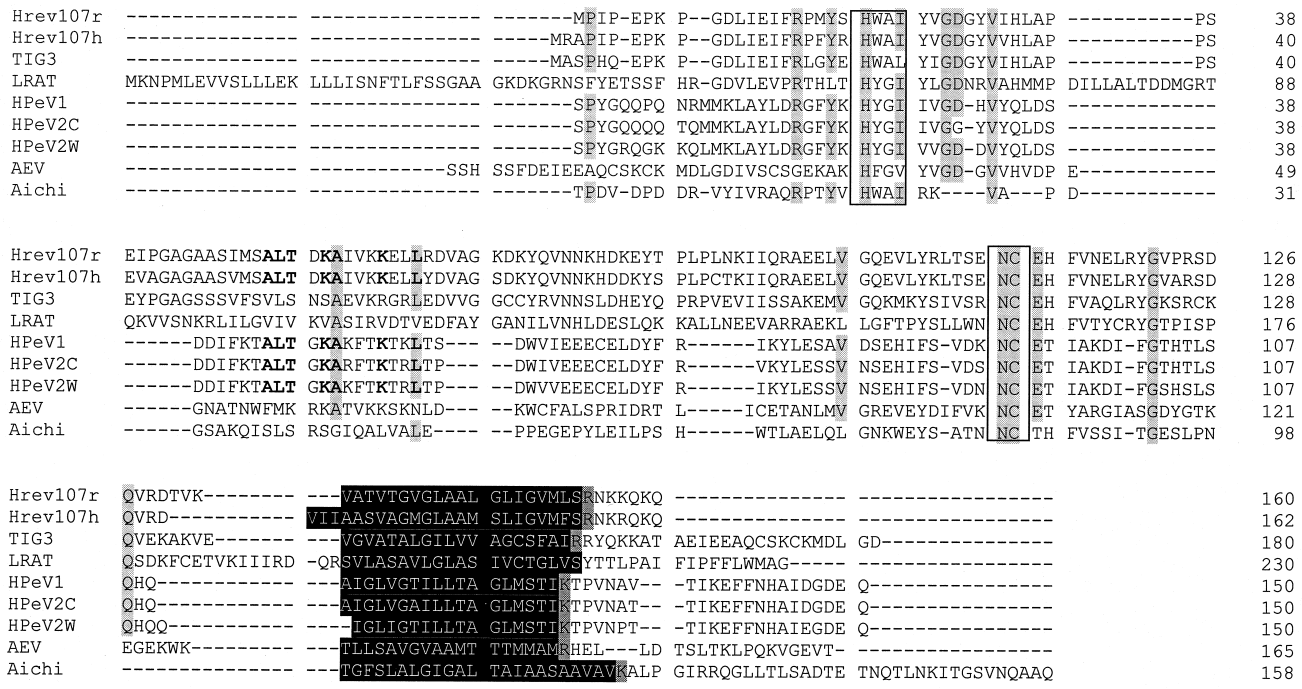

Fig. 1. Amino acid sequence alignment of the four cellular H-box/NC proteins and predicted 2A proteins of five picornaviruses. Residues seen in at least seven of the sequences are indicated by shading and the highly conserved H-box and NC regions are boxed. The transmembrane domain, predicted in all the sequences, is in white on black and the basic amino acid that follows this in most of the sequences is highlighted in dark grey. The sequence ALTXKAXXXKXXL, common to H-rev107 and human parechoviruses, is in bold. The two sequenced strains of HPeV2 are designated HPeV2W (Williamson) and HPeV2C [86-6760 (Connecticut/86)]. H-rev107r and H-rev107h are the rat and human homologues of this protein. The actual C-terminal boundary of the AEV 2A protein predicted here is not known. The level of significance of a match observed when comparing a sequence with a database can be assessed by the E value. This is the number of times the sequence is expected to match equally well as the observed match with a database of the same size containing random sequences. Close matches therefore have low E values. The E values of FASTA3-derived matches with the SWALL database for each protein subsequently introduced into this alignment were: AEV with TIG3 3·1, H-rev107h 4·3, HPeV1 4·3; HPeV1 with HPeV2C $1{\cdot}7 \times 10^{-54}$, HPeV2C 10^{-52}, AEV 5·6; HPeV2W with HPeV2C $1{\cdot}8 \times 10^{-53}$, HPeV1 $1{\cdot}9 \times 10^{-52}$; HPeV2C with HPeV1 $1{\cdot}8 \times 10^{-55}$, HPeV2W 10^{-54}; H-rev107r with H-rev107h $2{\cdot}1 \times 10^{-53}$, TIG3 $7{\cdot}7 \times 10^{-29}$, LRAT 9·9; H-rev107h with H-rev107r $5{\cdot}4 \times 10^{-55}$, TIG3 2×10^{-29}, LRAT 9·7; LRAT with TIG3 0·13, H-rev107r 6·1, H-rev107h 8·4. None of the other matches fell below the cut-off level of E = 10, but there is a clear relationship between the proteins shown in the highlighted areas, which define the H-box/NC proteins.

similar overall length. The HPeV1 position 24 region, hereafter termed the H-box, is seen in two forms among these proteins: HWA(I/L) in human and rat H-rev107, TIG3 and Aichi virus 2A; and H(Y/F)G(I/V) in HPeV1/2 and AEV 2A, together with LRAT. In addition, although there were no other absolutely conserved amino acids, the nature of some flanking residues is conserved and a Gly–Asp (GD) dipeptide is seen in most of the proteins. The NCE region is less well conserved in Aichi virus 2A and only Asn–Cys (hereafter termed the NC motif) is seen. The residues flanking the Aichi virus H-box are also less closely related to those of the other proteins. However, even in this virus, the significance of the similarity is emphasized by the presence of the hydrophobic region downstream of the NC motif. Moreover, there is clear primary sequence identity between the Aichi virus 2A and all the cellular proteins in the NC motif region, where the common sequence NCXHFV is seen.

The other characteristic feature of the virus and cellular proteins is the long (18–24 amino acids) hydrophobic region. According to the predictive program TMpred, this is highly likely to be a transmembrane domain (Hofmann & Stoffel, 1993). Deletion of this region has been shown to reduce the activity of H-rev107 severely, suggesting that it plays a key role in the function of this protein (Sers *et al.*, 1997). In all the proteins, except LRAT, this putative transmembrane domain is followed immediately by a basic amino acid. A further notable similarity is that seen between the H-rev107 proteins and HPeV 2A, where the motif ALTXKAXXXKXXL (positions 47–57 in HPeV1) can be seen in analogous positions.

In terms of overall amino acid identity, the H-rev107 and TIG3 proteins are closely related, while the other cellular protein, LRAT, is relatively distant from these. This is also evident from the alignment, which shows that LRAT has a longer N-terminal region and two relative insertions compared with the other cellular proteins (Fig. 1). If these are removed from comparisons, LRAT clearly clusters with the cellular

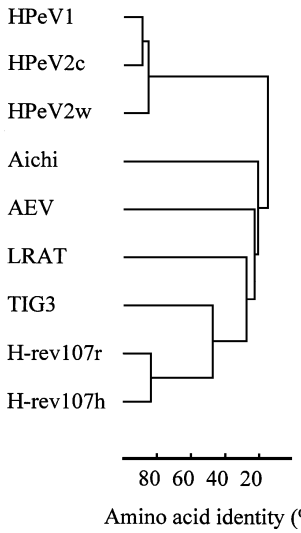

Fig. 2. Dendrogram, based on amino acid identities, expressing the relationship between the cellular H-box/NC proteins and picornavirus 2A proteins.

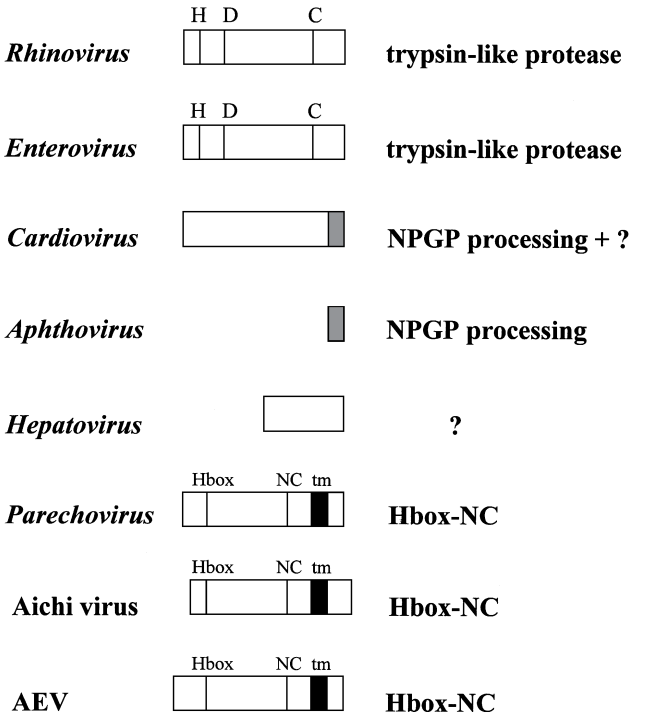

Fig. 3. Protein 2A types among the six picornavirus genera, together with Aichi virus and AEV. Aichi virus has been proposed as the type member of a new picornavirus genus, while in other parts of the genome the closest relatives of AEV are the hepatoviruses. Members of two other proposed new picornavirus genera (ERBV and PTV1) are aphthovirus-like in terms of 2A (King *et al.*, 1999). H, D and C refer to the catalytic triad of trypsin-like proteases, while Hbox, NC and tm (transmembrane domain) are the characteristic motifs of the H-box/NC proteins defined here. The hepatovirus 2A is of unknown function, while in addition to sequences associated with NPGP processing (shaded), the cardiovirus 2A has an additional region of unknown function. The actual C-terminal boundary of the AEV 2A protein predicted here is not known.

proteins, but remains a distant member (Fig. 2). Among the virus proteins, as expected, those from the human parechoviruses form a tight cluster.

If, as seems likely, the sequence identities between these proteins are indicative of shared functional properties, the strong conservation of the H-box, NC motif and putative transmembrane domain, within relatively poorly conserved proteins, leads to the prediction that these are critical functional elements within this class of protein. These features, which do not correlate with those of any other known proteins when subjected to PROSITE analysis, are therefore interesting targets for future work on both the virus proteins and their cellular homologues. His and Cys are two of the three amino acids involved in the catalytic triad of the 3C-like proteases seen in several viruses, including the 2A protein of rhinoviruses and enteroviruses (Ryan & Flint, 1997). Moreover, the conserved His and Cys residues within the H-box/NC proteins are similarly located to their counterparts in these proteases (Fig. 3). However, the amino acid sequence flanking the active site nucleophile of trypsin-like proteases (Cys in rhinovirus and enterovirus 2A) is characteristically Gly–X–Cys–Gly (GXCG), and the H-box/NC proteins do not conform to this pattern. His and Cys are also critical amino acids of papain-like proteases, such as the aphthovirus L, and of pestivirus p20 proteases, but once again there is no similarity of flanking amino acids in the H-box/NC proteins (Ryan & Flint, 1997; Ryan *et al.*, 1998). These observations suggest that the H-box/NC proteins are not proteases of a known type; indeed, it has already been shown that the HPeV1 2A has no proteolytic activity directed to either its own N or C terminus (Schultheiss *et al.*, 1995). The identification of LRAT, an enzyme responsible for the conversion of all-*trans*-retinol into retinyl esters, as a putative member of the H-box/NC proteins further suggests a non-proteolytic role for the virus proteins (Ruiz *et al.*, 1999). However, a proteolytic function cannot be ruled out.

The function of the cellular H-box/NC proteins may give a clue to the role of virus proteins in replication, but unfortunately there is little information on how these proteins exert an effect at the mechanistic level. This is compounded by the fact that the H-box and NC motifs have not been recognized previously and are not seen in any well-characterized proteins. It is known, however, that H-rev107 is down-regulated in a number of tumour cells and that overexpression leads to inhibition of cell proliferation and resistance to transformation by the ras oncogene homologue (Hajnal *et al.*, 1994; Sers *et al.*, 1997; Husmann *et al.*, 1998). This suggests that H-rev107 may act as a negative regulator of oncogenic ras signals, possibly by binding to and inhibiting an effector molecule (Husmann *et al.*, 1998). Expression of TIG3, which is induced by retinoic acid, likewise reduces cell proliferation (DiSepio *et al.*, 1998). Accordingly, it may contribute to the known inhibition of cell growth by retinoic acid. Both H-rev107 and TIG3 have been described as class II tumour

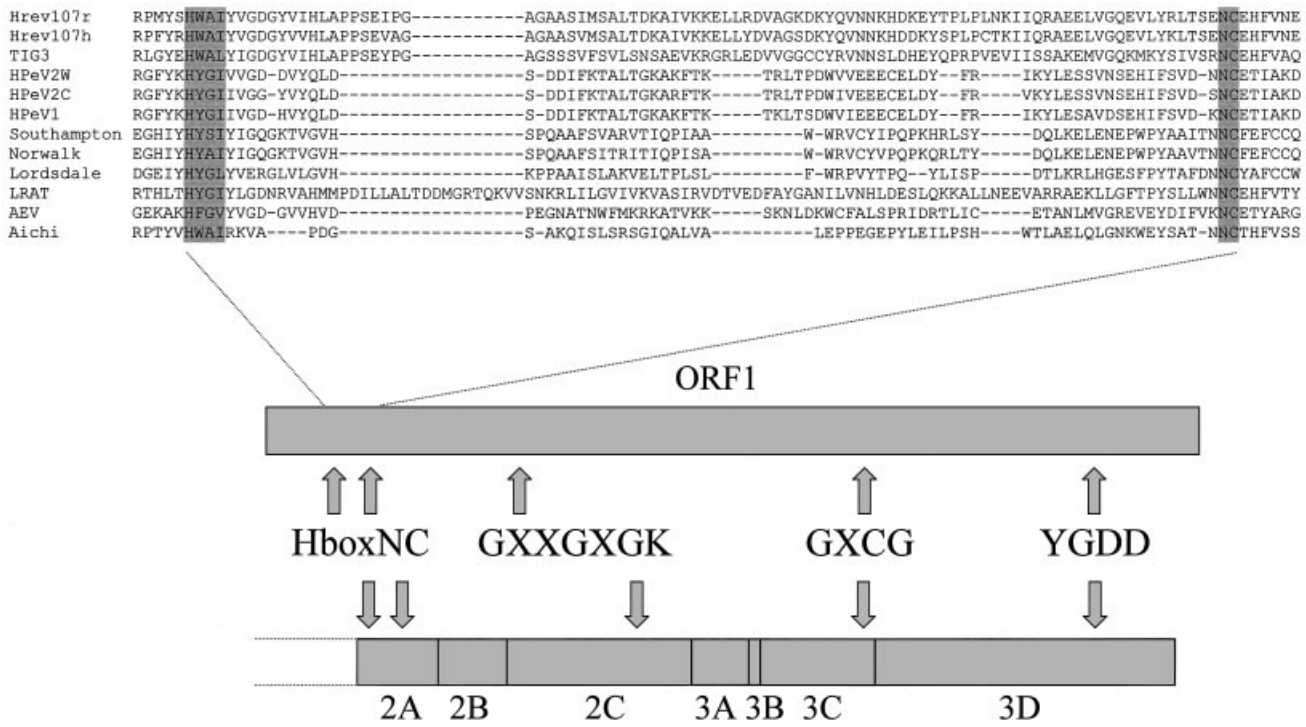

Fig. 4. Alignment of part of the cellular and picornavirus H-box/NC proteins with part of the non-structural region (ORF1) of caliciviruses of genetic group II (Southampton, Norwalk and Lordsdale viruses). The inclusion of the caliciviruses in the alignment is based on the observation, by FASTA3 analysis of the SWALL database, of a match between H-rev107r and Norwalk virus ($E = 2{\cdot}0$). A subsequent search, using the region of Norwalk virus sequence shown in the figure, indicated, as expected, a close relationship with two other caliciviruses, Southampton virus ($E = 1{\cdot}1 \times 10^{-32}$) and Lordsdale virus ($E = 3{\cdot}7 \times 10^{-12}$). The whole non-structural region of the picornavirus HPeV1 is also aligned schematically with that of ORF1 of the calicivirus Lordsdale virus, in order to demonstrate the similar arrangement of non-structural proteins.

suppressors. These are proteins that are expressed at very low levels in cell lines or tumours, permitting cell proliferation, even though their genes are intact (Husmann *et al*., 1998). As already discussed, LRAT was identified on the basis of enzymic activity, the conversion of all-*trans*-retinol into retinyl esters, which is an important pathway in the metabolism and storage of retinol (Ruiz *et al*., 1999). It appears to be unrelated to known retinol-binding proteins and to other acyltransferases, suggesting that it functions in a distinct manner. However, the association with retinoids may again suggest a role in the control of cell growth. If the virus H-box/NC proteins have a similar effect on cell proliferation, this may be a mechanism, for example, for reducing host-cell macromolecular synthesis and may therefore serve as an alternative to the host-cell protein synthesis shut-off induced by some picornaviruses. On the other hand, the virus proteins may be involved in phenomena such as the regulation of apoptosis, as proteins capable of inducing or suppressing this process have been identified in several viruses. The implicit identification of their critical features, through the recognition of the H-box/NC protein family, gives pointers to how these questions may be addressed.

From its earliest isolation, it was observed that HPeV1 causes a distinctive cellular pathology, involving characteristic nuclear changes (Wigand & Sabin, 1961; Shaver *et al*., 1961; Jamison, 1974). The report that H-rev107 associates with the nuclear membrane, together with the relationships described here, raises the intriguing possibility that this pathology results from the action of the 2A protein (Sers *et al*., 1997).

It is assumed that many virus proteins originated by acquisition from the host genome. In the case of picornaviruses, the structural similarity of 3C (and that of the related entero/rhinovirus 2A protein) to cellular trypsin-like proteases is cited as an example (Gorbalenya, 1992). The papain-like, aphthovirus L protein may also have arisen from capture of a host RNA. The presence in coxsackievirus A9 of an Arg–Gly–Asp (RGD)-containing, C-terminal VP1 extension (relative to other enteroviruses), with similarity to transforming growth factor $\beta 1$, is a more speculative example of a region of a picornavirus protein with a putative cellular origin (Chang *et al*., 1989, 1992; Hughes *et al*., 1995). Apart from these cases, the observation reported here, that some picornavirus 2A proteins have cellular homologues, is the clearest evidence in picornaviruses for such a mechanism.

The demonstration that the 2A proteins of three picornaviruses are related reduces the apparent diversity of this locus and, as shown in Fig. 3, picornaviruses fall essentially into four

groups. These are viruses with: a trypsin-like protease (rhinoviruses/enteroviruses); an NPGP motif involved in 2A/2B cleavage (cardioviruses/aphthoviruses); a 2A unrelated to known proteins (hepatoviruses); or an H-box/NC protein (parechoviruses/Aichi virus/AEV). Although the C terminus of the cardiovirus 2A is homologous to the aphthovirus protein, it has an additional 130 N-terminal amino acids. The occurrence of an H-box/NC protein at the same locus in three diverse picornavirus groups suggests that it was acquired by a single event in a common ancestor and that it has been maintained through subsequent divergence. Since AEV is related to hepatoviruses, which lack a corresponding protein, it is likely that hepatoviruses lost this protein after divergence from the AEV lineage. This may have taken place through drift, or through deletion and subsequent capture of another protein-encoding sequence at this locus. In view of the distinct nature of the 2A proteins of AEV and hepatoviruses, it may be preferable to consider them as members of separate genera, notwithstanding the fact that they are more closely related to one another than to other picornaviruses in other parts of the genome (Marvil *et al.*, 1999). Recently, a parechovirus-like virus has been isolated from rodents (Nicklasson *et al.*, 1998, 1999). It will be interesting to ascertain whether this virus has an H-box/NC protein and, if so, how closely related it is to that of HPeVs.

Relatively diverse picornaviruses have H-box/NC proteins and it is possible that they occur in other viruses. It was therefore interesting to find similar features in one genetic group of the caliciviruses (group II), containing Norwalk, Southampton and Lordsdale viruses, by comparison with H-rev107 sequences (Lambden *et al.*, 1993; Dingle *et al.*, 1995; Hardy & Estes, 1996). These viruses each have sequences reminiscent of the H-box and also possess an NC motif (Fig. 4). Caliciviruses have a similar relative arrangement of non-structural proteins to picornaviruses, which can be summarized: NTP-binding protein (possibly helicase); 3C-like protease; polymerase (2C; 3C; 3D in picornaviruses). The calicivirus H-box/NC protein is upstream of the NTP-binding protein and the relative positions of the motifs are similar to those of the picornavirus protein (Fig. 4). The calicivirus protein is distinguished from the picornavirus 2A by the absence of a significant transmembrane domain, suggesting that it may function in a different manner. However, it seems that H-box/NC proteins may occur in several RNA viruses, suggesting that they may contribute an important, but as yet unknown function to the virus life-cycle.

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