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The detection of subtle differences between different orthopoxvirus genomes by heteroduplex analysis

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Summary

Corresponding DNA fragments from variola (Harvey) and monkeypox (Denmark) viruses which had been cloned into different plasmid vectors were subjected to heteroduplex analysis. Characteristic deletion loops corresponding to differences between the cloning vectors served as internal markers to identify and to orientate the heteroduplexed molecules. Partial denaturation of the resulting heteroduplexes was used as a primary screen to locate regions of heterogeneity between the poxvirus inserts. The denaturation threshold for homoduplexes was consistently higher than that for heteroduplexes. However, significant sequence divergence between corresponding fragments was indicated by larger than usual differences in thresholds between corresponding homo- and heteroduplexes. Denaturation bubbles of 0.1–0.5 kb were detected and hence small regions of heterogeneity between the genomes (180 kb) of variola and monkeypox viruses were localised. This procedure has a general application in comparative studies on large, complex but closely related DNA molecules.

cloning, DNA, electronmicroscopy, heteroduplex, monkeypox, partial denaturation, variola

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Introduction

There is an unidentified animal reservoir of monkeypox virus in the tropical rain forest belt of West and Central Africa. In this region occasional, sporadic human infections with this virus have been reported. These clinically resemble smallpox, although sustained transmission of monkeypox virus in man has not been reported and monkeypox does not pose a serious public health problem. Nevertheless, there is a need to identify the differences between monkeypox virus and variola virus as part of the surveillance policy following the eradication of smallpox.

The cleavage sites for the endonucleases *Hin*dIII and *Xho*I on the genomes of a number of orthopoxviruses have been mapped (Mackett and Archard, 1979). The positions of these cleavage sites were strongly conserved, particularly in the central 60–70% of the genome. There were some differences between the genomes of monkeypox and variola which were constant over several isolates of each virus even within the conserved region. However, corresponding fragments from these genomes cross-hybridize strongly, showing that they consist of closely homologous DNA sequences (Mackett and Archard, 1979). Hence a different approach is necessary for the location of short, non-homologous sequences within the genomes of monkeypox and variola.

One such approach is the examination of heteroduplexes of corresponding DNA fragments. Deletions or totally non-homologous sequences of about 100 nucleotides and upwards can be detected and would appear as either loops or bubbles respectively in heteroduplexed DNA molecules. Initial work comparing cloned fragments of DNA from monkeypox with corresponding fragments from variola showed that heteroduplex analysis alone did not detect significant differences between these fragments. The work was extended to the analysis of partial denaturation patterns, which in homoduplexed DNA molecules produces bubbles within regions rich in adenine and thymine due to the lower melting temperatures of such sequences. In heteroduplexed DNA molecules short sequences of mis-matched bases will also contribute to a lowering of the melting temperature in localised regions and will thereby generate bubbles on partial denaturation (Davis et al., 1971; Garon et al., 1973; Kudler et al., 1983). A comparison of denaturation thresholds between corresponding homo- and heteroduplexes and the positions of the bubbles produced should therefore localise small regions of heterogeneity.

In order to refine this analysis, and as an aid to the identification of heteroduplexes, variola DNA fragments were cloned into the plasmid pAT153 whereas monkeypox DNA fragments were cloned into the plasmids pBR328 and pBR329. Deletion loops corresponding to differences between these plasmids identified and oriented heteroduplexed DNA molecules and also served as internal markers for the localisation of the denaturation bubbles within the corresponding poxvirus fragments.

Although this procedure has been used to compare and identify regions of heterogeneity in the genomes of two different orthopoxviruses it is also applicable for locating precisely, and relatively quickly, subtle differences between other large but closely homologous DNA molecules.

Materials and Methods

Virus DNA

Variola virus, strain Harvey, and monkeypox virus, strain Denmark, were grown on chick chorioallantois and the virus was extracted and purified by differential centrifugation. DNA was extracted from purified virions by lysis in detergent, digestion with protease and phenol/chloroform extraction. Further details are given by Sam and Dumbell (1981) and Mackett and Archard (1979).

Recombinant DNA

Variola or monkeypox DNA was digested with *Hin*dIII and the resulting fragments inserted into either pAT153 or pBR328 and pBR329 respectively, using standard cloning procedures. Recombinants were identified using restriction enzyme digestion and hybridization (Dollery et al., in preparation). Selected recombinant plasmids were grown in *E. coli* strain HB101 and purified by isopycnic banding in caesium chloride gradients. Recombinant plasmids used for heteroduplexing and partial denaturation were linearized with an excess of a restriction endonuclease chosen so that a single cut would be made in the vector component. Digestion was at 37 °C for 45 min in a total volume of 50 μl; the reaction was stopped by adding 2% SDS solution. After 5 min at room temperature, protein was removed by chromatography on Sepharose 2B (4 cm × 0.5 cm): elution was with 250 μl (5 × 50 μl) of 10 mM Tris-HCl, pH 8.5/1 mM Na_2EDTA. The last 100 μl contained approximately 60–70% of the DNA applied to the column. The protein-free solutions of linearized DNA were stored at 4 °C.

Heteroduplex formation and partial denaturation

Equimolar amounts of linearized recombinant plasmids (200 ng of each) containing either monkeypox or variola DNA inserts were mixed and denatured in 100 mM NaOH/20 mM Na_2EDTA (total volume 22.5 μl) for 10 min at room temperature (Davis et al., 1971). The solution was neutralized by addition of 100 mM Tris-HCl, pH 7.2, and 50% formamide to give a final volume of 50 μl. The fragments were allowed to reanneal at 33 °C for 5–60 min depending on their length; under these conditions approximately 50% of the molecules were double-stranded. The hyperphase was then prepared. Typically this contained 25 ng of the heteroduplexed DNA, 25 ng of single-stranded φX174 DNA, 25 ng of double-stranded PM2 DNA, 5 μg cytochrome *c* (type V, Sigma), in 100 mM Tris-HCl, pH 8.5, 10 mM Na_2EDTA and formamide at a specified concentration in the range of 40–80% to a total volume of 50 μl. The DNA was spread at 30 °C in a perspex hood onto the hypophase containing 10 mM Tris-HCl, pH 8.5, 1 mM Na_2EDTA and formamide at a concentration 30% less than in the hyperphase. The same conditions of salt concentrations and temperature were used in each experiment; slight changes in either of these changed the threshold concentration of formamide at which strand separation was first detected. Similarly, strict controls of the 'isodenaturing' conditions between the hyperphase and hypophase were maintained (Davis and Hyman, 1971). The cytochrome film was picked up on a collodion-coated grid (Kleinschmidt,

1968), stained with uranyl acetate in 90% alcohol, rinsed in 90% alcohol, dried in 2-methylbutane, shadowed with gold (40%)–platinum (60%) on a rotating stage and coated with carbon. The grids were viewed in a Phillips 300 electron microscope and at least 30 molecules were photographed for each threshold. Base mismatch was demonstrated by comparing the threshold formamide concentration for denaturation in the heteroduplexes with that for the corresponding homoduplexes.

Results

The *Hin*dIII DNA fragments of variola (strain Harvey) were cloned in *E. coli* using the vector pAT153 whereas those of monkeypox (strain Denmark) were cloned using both pBR328 and pBR329 vector. The *Hin*dIII cleavage site maps for both variola and monkeypox (Mackett and Archard, 1979) are reproduced in Fig. 1. The fragments shown in large letters are those used in the studies presented here; taken together, they account for approximately 25% of each virus genome and about 40% of the central conserved region. Recombinant plasmids in which the equivalent fragments of the two viral genomes were inserted in the same orientation with respect to the vector sequences were used for this analysis. These orientations were confirmed by endonuclease mapping.

Heteroduplexes between pAT153 and pBR328 (pBR329)

The plasmid cloning vectors pAT153 (Twigg and Sherratt, 1980) and pBR328 (Soberon et al., 1980) were each derived from pBR322 (Bolivar et al., 1977) and their relationships are shown in Fig. 2. They differ from each other in two respects. Firstly, pBR328 has an additional sequence of 1633 nucleotide pairs which contains the gene for chloramphenicol transacetylase. Secondly, the two plasmids have non-identical deletions of sequences which are present in pBR322 and are concerned with plasmid mobilization. The shorter deletion is in pAT153 and results in that plasmid having an additional sequence of 384 nucleotide pairs which are absent in pBR328 and pBR329. A heteroduplex between pAT153 and pBR328, each linearized at their single *Pst*I cleavage site, is shown in Fig. 3a where the position of the

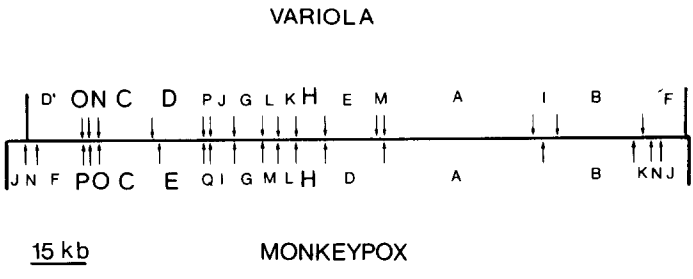

Fig. 1. Physical map locations of the *Hin*dIII fragments of monkeypox and variola DNA (Mackett and Archard, 1979). The fragments shown in large letters indicate those used for the studies presented here.

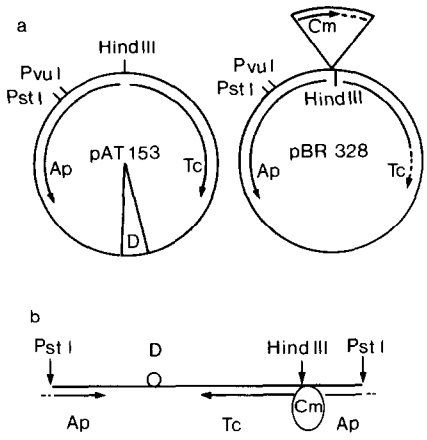

Fig. 2. (a) The relationship between pAT153 and pBR328. Cm represents the sequence containing the chloramphenicol transacetylase gene which is present only in pBR328; D represents the smaller sequence absent in pBR328 but present in pAT153. The broken lines indicate the two identical sequences of part of the tetracycline resistance gene (Tc) in pBR328; in pBR329 the tetracycline sequence associated with the chloramphenicol transacetylase gene has been removed; Ap denotes the Ampicillan resistance gene. (b) Heteroduplex between the two plasmids pAT153 and pBR328 both linearised at the *Pst*1 site and showing the large and small deletion loops. The *Hin*dIII cloning site is 24 nucleotides from the large Cm deletion loop.

two single-stranded deletion loops can be seen clearly. These structures can be used as internal markers to identify and orientate heteroduplexed molecules.

The large 1633 base pair loop in pBR328 contains a 482 base pair inverted duplication of part of the tetracycline resistance gene (Prentki et al., 1981; Fig. 2). Stem loop structures formed by rapid intra-strand reannealing of these sequences were observed in 30–50% of the molecules which contained pBR328. This inverted duplication is absent in pBR329 and use of this vector in later experiments avoided the complication of stem loop structures.

Denaturation of homo- and heteroduplexed recombinant DNA molecules

Strand separation of homo- and heteroduplexed DNA was achieved by increasing the formamide concentration in the spreading mix. This resulted in a lowering of the melting temperature for strand separation in double-stranded DNA molecules: a 1% increase in formamide concentration decreases the melting temperature by 0.5 °C. The threshold formamide concentration at which strand separation was first observed in heteroduplexed DNA molecules was dependent on the percentage of adenine and thymine in each strand and on the degree of base mis-match between the two complementary strands. The threshold formamide concentrations for strand separation in any heteroduplex was compared with those for the corresponding homoduplexes so that the extra differences which reflect base mis-match could be assessed.

Typical homoduplexes of recominant plasmids denatured at different formamide concentrations are shown in Figs. 3b, c and d. These figures relate specifically to the

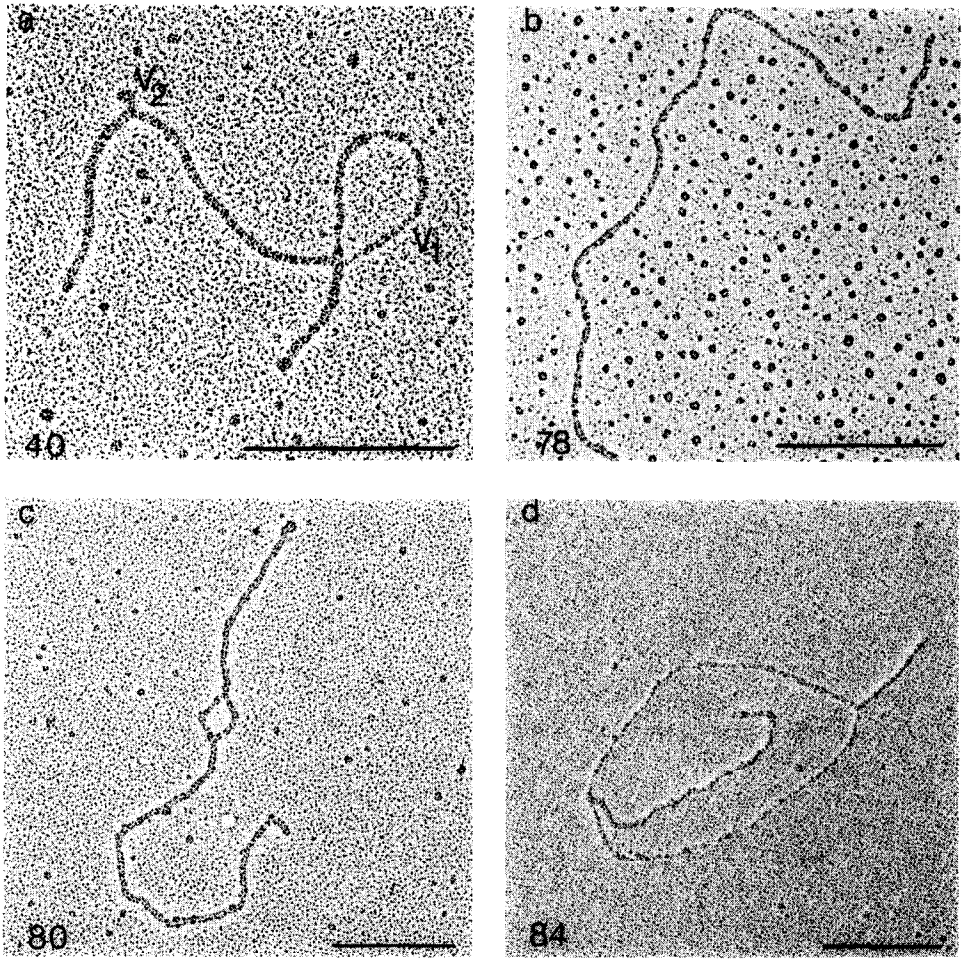

Fig. 3. (a) A heteroduplex between the cloning vectors pAT153 and pBR328 (the plasmids were linearized with *Pst*1). The large and small single-stranded deletion loops are labelled V_1 and V_2, respectively. The smaller loop frequently collapses to form a spike structure. The bar in all the figures represents 1 kb. The number in the lower left-hand corner of each micrograph represents the percent formamide in each spreading mix (hyperphase). (b–d) Homoduplexes of pAT153 containing an insert of variola *Hin*dIII N and showing the structures produced by increasing the concentration of formamide in the hyperphase. The threshold formamide concentration for denaturation of variola *Hin*dIII N is given as 80% (c). When the hyperphase contains 84% formamide the insert is denatured completely (d).

recombinant plasmid containing the Harvey *Hin*dIII N insert. Completely double-stranded molecules were obtained when the formamide concentration in the hyperphase was below 78% (Fig. 3b). In contrast, at formamide concentrations of 84% the insert was completely denatured into single strands which were held together by the flanking duplex tails of vector DNA (Fig. 3d). These structures were obtained because orthopoxvirus DNA has a higher adenine plus thymine content (70%) than the vector DNA (50%). Denaturation of the vector was not observed within the

range of formamide concentrations used in these experiments. Spreading the DNA at intermediate concentrations of formamide resulted in partially denatured molecules (Fig. 3c) with the single-stranded bubbles corresponding to regions which can be presumed to be richer in adenine and thymine than other parts of the same molecule. The threshold formamide concentration for strand separation (denaturation) varied according to the recombinant plasmid (and hence the poxvirus insert) under investigation; the positions of the denaturation bubbles also varied.

The threshold formamide concentrations for strand separation of the heteroduplexes formed between recombinant plasmids containing each of five equivalent fragments from variola and monkeypox DNA are shown in Table 1. These concentrations were consistently lower than those for the corresponding homoduplexes. For three of the pairs of fragments the heteroduplexes began to denature at formamide concentrations approximately 10% lower than those for the corresponding homoduplexes. In contrast, the denaturation threshold for heteroduplexes between variola *Hin*dIII N and monkeypox *Hin*dIII O was some 23% lower than the concentration required for the corresponding N and O homoduplexes. The partially denatured heteroduplex between variola *Hin*dIII N and monkeypox *Hin*dIII O is shown in Fig. 4a; strand separation first occurred at 57% formamide. Complete denaturation of the insert occurred at 61% formamide (Fig. 4b).

In order to assess whether there was any 'peel-back' effect from the large deletion loop which is adjacent to the *Hin*dIII site used for insertion (Fig. 2), monkeypox *Hin*dIII O was cloned into both pAT153 and in pBR328 and then heteroduplexed. As strand separation was not observed at 57% formamide (Fig. 4c), it was concluded that the large deletion loop resulting from the difference between pAT153 and pBR328 played no role in determining the denaturation threshold observed with the variola monkeypox heteroduplexes. This conclusion was confirmed by determining the threshold formamide concentration for denaturation of heteroduplexes between variola *Hin*dIII N and monkeypox *Hin*dIII O when each fragment was inserted in pAT153 (Fig. 4d). The concentration of formamide for strand separation was again

TABLE 1

FORMAMIDE CONCENTRATIONS FOR THE DENATURATION OF HETERODUPLEXES AND CORRESPONDING HOMODUPLEXES

	Pairs of equivalent fragments $\frac{\text{variola}}{\text{monkeypox}}$ (size in kb)				
	$\frac{D}{E}$ (15)	$\frac{H}{H}$ (8,5)	$\frac{N}{O}$ (2,2)	$\frac{O}{P}$ (1,5)	$\frac{C}{C}\ \frac{15.6}{17.7}$
Threshold concentration of formamide for strand separation in:					
Variola homoduplexes	66	68	80	77	64
Monkeypox homoduplexes	68	65	78	76	65
Variola/monkeypox heteroduplexes	58	58	57	67	48
Threshold difference between homo- and heteroduplexes	8–10	7–10	21–23	9–10	16–17

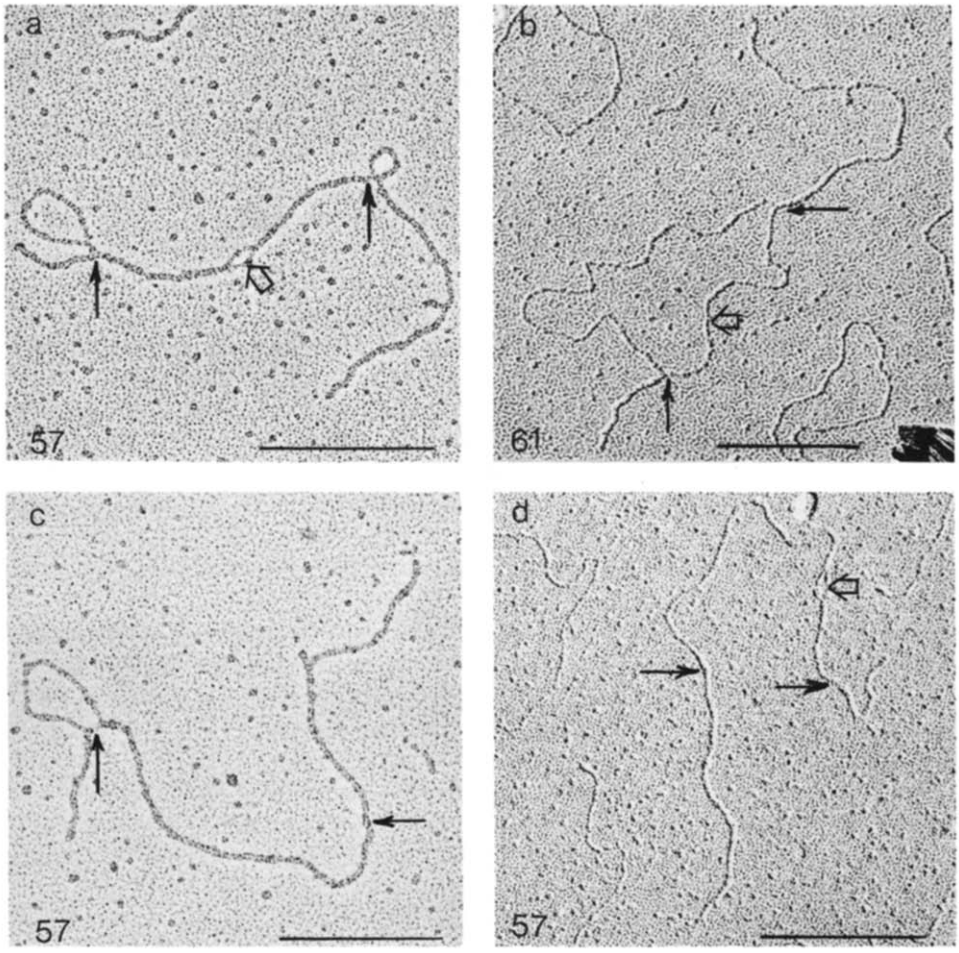

Fig. 4. (a) Heteroduplex between variola *Hin*dIII N in pAT153 and monkeypox *Hin*dIII O in pBR328. The recombinant plasmids were linearized by digesting with *Pvu*I. Denaturation bubbles (open arrows) are first observed within the poxvirus insert when the formamide concentration is 57%. The approximate position of the insert in each micrograph is indicated by the solid arrows and single strands are indicated by open arrows. (b) When the hyperphase contains 61% formamide the poxvirus insert is completely denatured. One of the single strands of the bubble is longer than the other because it is composed of monkeypox *Hin*dIII O and the large single-stranded deletion loop in the vector. (c) A heteroduplex formed between the same insert (monkeypox *Hin*dIII O) in different vectors (pAT153 and pBR328). (d) A heteroduplex formed between the two different inserts (variola *Hin*dIII N and monkeypox *Hin*dIII O) in the same vector (pAT153).

57% and it was therefore concluded that these threshold values were independent of vector DNA sequences.

Heteroduplexes between the *Hin*dIII C fragments of each virus were also investigated. This requires special consideration as monkeypox *Hin*dIII C is approximately 2 kb longer than variola *Hin*dIII C (see Fig. 1). Most of the surplus sequences within

monkeypox *Hin*dIII C were detected as a large single-stranded loop in heteroduplexes with variola *Hin*dIII C when these were spread at low formamide concentration (Figs. 5a and b). The position of this large loop (I_1) divides the

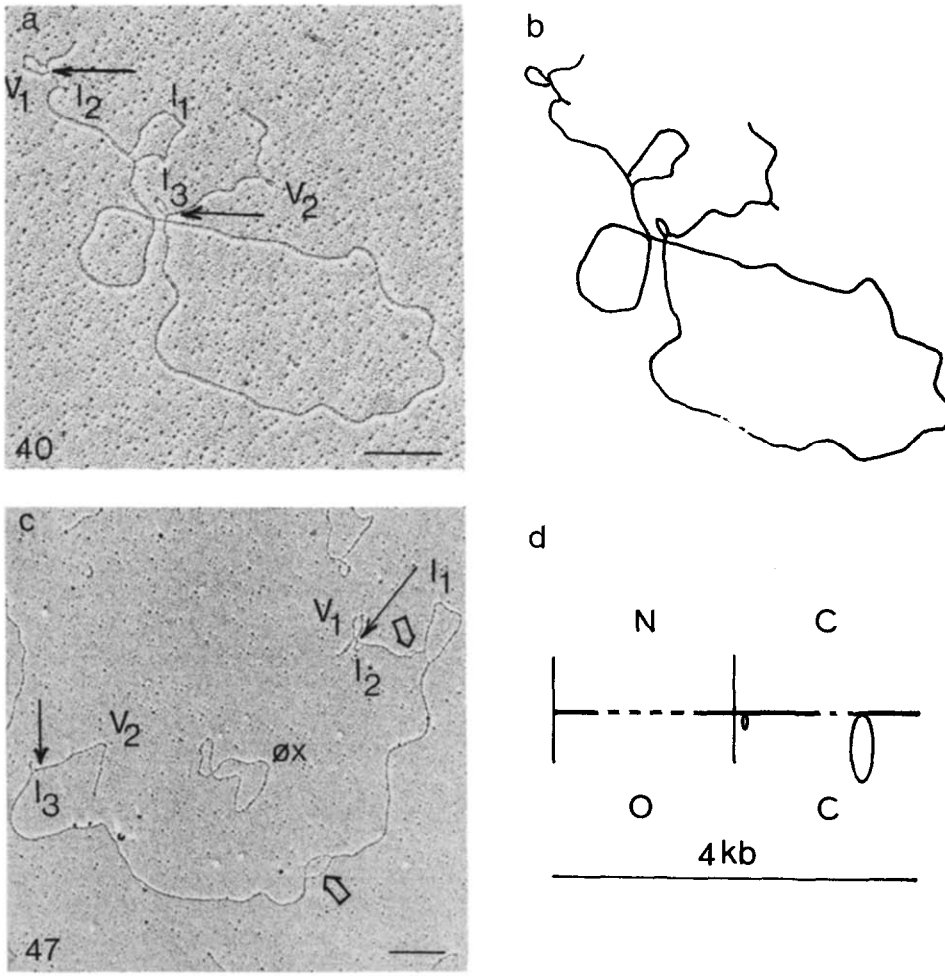

Fig. 5. (a) Heteroduplex between variola *Hin*dIII C in pAT153 and monkeypox *Hin*dIII C in pBR329. These recominant plasmids were linearized by digestion with *Pvu*I. V_1 and V_2 are the large and small deletion loops formed between heteroduplexes of pAT153 and pBR329 (V_1 represents a deletion smaller than that between pAT153 and pBR328). I_1, I_2 and I_3 are single-stranded deletion loops formed by heteroduplexing the two *Hin*dIII C inserts. (b) Tracing of molecule illustrated in a. (c) The threshold formamide concentration for denaturation of the variola *Hin*dIII C and monkeypox *Hin*dIII C is 47%. Two regions of heterogeneity are indicated by the open arrows. Solid arrows indicate the extent of the orthopoxvirus inserts. The small vector deletion loop (V_2) is absent in this spread. (d) Schematic representation to show the structure of the region of heterogeneity which extends from the pair variola *Hin*dIII N/*Hin*dIII O monkeypox *Hin*dIII O into the left-hand end of the *Hin*dIII C fragments of both viruses (cf. Fig. 1). Dotted lines represent significant base mis-match demonstrated by partial denaturation; deletions are indicated by loops.

heteroduplexed *Hin*dIII C fragments into two unequally sized regions: a shorter sequence of approximately 2 kb which is at the left hand end (in relation to the map shown in Fig. 1), and which is adjacent to the large vector loop (V_1) and a larger sequence (approximately 13.6 kb) to the right. In addition, two small single-stranded loops (I_2, I_3) were detected (Figs. 5a, b and c) which indicate surplus sequences in either the variola or the monkeypox DNA. These small insert deletions (I_2, I_3) are placed approximately 0.2 kb and 1.5 kb respectively from the large (V_1) and small (V_2) vector deletions (Fig. 5a). These data indicate that the difference in the position of the *Hin*dIII site which separates variola *Hin*dIII C and D and monkeypox *Hin*dIII C and E is caused either by a translocation event or by the replacement of a region of monkeypox *Hin*dIII C by an unrelated sequence and not by, for example, a double mutation.

The left hand 2 kb sequence of the heteroduplexed *Hin*dIII C fragments contained sequences adjacent to variola *Hin*dIII N or monkeypox *Hin*dIII O, and was shown to have a threshold formamide concentration for denaturation of 47% (Fig. 5c; Table 1). A short region having a similar threshold also was observed within the larger flanking segment (Fig. 5c; Table 1). The denaturation thresholds for the corresponding homoduplexes were 65% formamide; a difference of 17% which indicated significant heterogeneity. In contrast, the remainder of the heteroduplexed insert did not denature until a formamide concentration of 56% was reached.

Reference to Fig. 1 will show that just as monkeypox *Hin*dIII C was longer than variola *Hin*dIII C so was variola *Hin*dIII D longer than monkeypox *Hin*dIII E by an equivalent amount. However, locating surplus sequences of variola in this pair of fragments has not been entirely satisfactory. The reason for this is that no endonuclease was available which cut the vector sequences and not the insert to linearize the recombinant plasmid, as was done for the other pairs. It was necessary to excise the inserts with *Hin*dIII and to heteroduplex those without 'tails' of vector DNA. In the spreads, heteroduplex molecules had a single-strand DNA sequence at one end and a small internal deletion. However, the insert had lost its orientation and it can only be deduced that the bulk of the surplus variola sequences were not located in the central part of variola *Hin*dIII D. Further work will be necessary to test, by hybridization, whether the surplus sequences of monkeypox and of variola in this region of the genome represent a translocation of a common sequence or two unrelated sequences.

Discussion

A method is described here by which large, complex but closely related DNA molecules may be screened for small regions of heterogeneity. The method involves the cloning of DNA fragments into different vectors and the subsequent partial denaturation of corresponding heteroduplexes.

The use of different vectors for cloning the two molecules to be compared is an essential part of the procedure. Firstly, it enables the heteroduplex molecules to be identified even when they form only a small proportion of the molecules present.

Secondly, the vector deletions act as internal marker from which the location of small denaturation bubbles can be measured. Heteroduplexed DNA was spread at a constant temperature of 30 °C. Previously it had been noted that small rises in ambient temperature would reduce the concentration of formamide needed to reach the denaturation threshold. The threshold formamide concentration is reduced by 2% formamide for each 1 °C rise in temperature. Similarly, the salt concentrations of the solutions used in consecutive experiments must be the same, for differences in these would also affect the threshold formamide concentrations for denaturation (Dove and Davidson, 1962). The use of the *Hin*dIII site for cloning the genomic fragments of these orthopoxviruses is historical. For heteroduplexing these recombinant molecules, this site is not ideal, as the end of the large vector deletion (V_1) is only 24 nucleotides away from the *Hin*dIII site. Although it has been assessed that the deletion loop formed between pBR328 (pBR329) and pAT153 does not lower artificially the denaturation threshold, viscous forces may cause a slight separation in some spreads. Cloning into the *Bam*H1 or *Pst*1 sites, for example, would eliminate any need to assess such an effect.

Pox virus sequences in heteroduplexed DNA molecules were completely denatured in formamide concentrations much lower than those required to denature the vector sequences. This reflects the relatively low adenine plus thymine content of each vector. Vector sequences did not affect the threshold formamide concentrations necessary for denaturation of pox virus DNA sequences. It was concluded that the denaturation patterns observed in the heteroduplexes can be used to locate regions of genetic heterogeneity in closely related DNA molecules.

The formamide concentrations necessary for denaturing poxvirus DNA sequences in homoduplexes varied between 64% and 80%. Presumably, this reflects variations in the proportion of adenine plus thymine in different regions of each virus genome. Such differences may occur within the length of a single fragment and hence it is probable that partial denaturation of heteroduplexes may be biased towards the detection of heterogeneity within the regions rich in adenine plus thymine. The ability to localise the denaturation bubble could, however, permit a second round of screening in which the formamide concentrations were raised above the threshold for the first bubble and compared also for the second or third bubble to appear in corresponding homo- and heteroduplexes. This, however, has not yet been attempted.

Heteroduplexes consistently had lower threshold formamide concentrations for denaturation than the corresponding homoduplexes. This was anticipated as during the evolutionary histories of monkeypox and variola, selective pressures for conservation of function would not necessarily preserve identical base sequences or eliminate mutations within any redundant sequences. It was concluded that the sequence divergence between the monkeypox and variola genome was not meaningful if the difference in the threshold formamide concentrations for denaturation of heteroduplexes and corresponding homoduplexes was 10% or less. Differences substantially greater than 10% were found with two pairs of fragments, which are adjacent in the intact genome. This is interpreted as a significant heterogeneity which extends from the pair variola *Hin*dIII N/monkeypox *Hin*dIII O into the

*Hin*dIII C fragments of both viruses. There is also a deletion loop, some 2 kb in length formed by surplus sequences located in the monkeypox genome approximately 2 kb from the *Hin*dIII site separating monkeypox *Hin*dIII O and *Hin*dIII C fragments and a deletion approximately 0.2 kb in length adjacent to the same *Hin*dIII site (Fig. 5d). Thus heteroduplex analysis covering 43–45 kb of the conserved region of the two genomes has pinpointed a region of 4–6 kb which contains a significant heterogeneity and on which a more detailed comparison should concentrate. It is worth noting that genes in host range restriction are encoded in the equivalent region of the rabbitpox strain of vaccinia virus (Moyer and Rothe, 1980). Other regions of heterogeneity may well be encountered when the rest of the monkeypox and variola genomes are subjected to heteroduplex analysis.

The more diverse monkeypox (Denmark) and variola (Harvey) prove to be, the more confidently can it be predicted that the eradicated virus, variola, could not again be derived by genetic alterations to monkeypox. This opinion would be further strengthened if sequences unique to variola (Harvey) were to be demonstrated.

Acknowledgements

Virus was grown under Category A containment conditions and cloning experiments were done under Category I containment conditions as specified by DPAG and GMAG, respectively. We thank Dr. H. Delius for discussions on electron microscopy, Dr. B. Griffin for constructive criticism of the manuscript, Mrs. L. Mann for providing some of the monkeypox clones, and Mr. A.B. Dowsett and Mrs. E. Elphick for technical assistance. This work was supported by the orthopox programme of the MRC.

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