DETECTION OF SEQUENCE HETEROLOGY BY USE OF THE N.CRASSA NUCLEASES

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SUMMARY: We have used the single-strand specific nucleases of Neurospora crassa to detect sequence divergencies between two similar DNA molecules: restriction endonuc lease EcoRI produced linears from Simian Virus 40 and a variant of human origin,DAR. Enzyme treatment of the heteroduplex DNA resulted in specific cleavage into two fragments of one-third and two-thirds genome length. These two viral DNAs therefore have at least one region of heterology located about 0.35 map units from the EcoRI site. Due to the known specificities of the N.crassa nucleases, this technique is applicable to detect mutations in RNA or DNA genomes.

Classical genetic mapping studies have in recent years been supplemented by the use of biochemical and physical techniques. Assessment of the relatedness of two polynucleotide genomes can be performed in a variety of ways, such as hybridization studies (1) or electron-microscopic heteroduplex mapping (2). We report here a novel technique for the localization of sequence heterologies, due to divergence or mutation, that has applicability to any DNA or RNA genome. It makes use of the N.crassa single-strand specific nucleases (3,4,5,) to detect mismatched sequences that are too small to be identified by most other methods.

The enzymes used are highly single-strand specific (6,7,), and will degrade denatured DNA or RNA equally well (8). They have been used to eliminate large mismatched regions from Adenovirus heteroduplex DNA molecules, yielding specific fragments (9) , and were shown to detect sequence heterologies below the limit of electron microscope visualization (10). Small weakly hydrogen-bonded regions in superhelical DNAs were cleaved by these enzymes (7) . Furthermore, the single - stranded loops of $tRNA^{Phe}$ are sensitive to the N.crassa endonuclease (8) as are

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distortions in duplex DNA due to thymine dimers introduced by ultraviolet irradiation (11). The DNAs were from the mammalian transforming virus SV40[1] and DAR (12), an SV40 variant of human origin (also referred to as the PML-2 strain(13) . This latter papovavirus was originally isolated from a patient with PML (progressive multifocal leukoencephalopathy, a rare subacute demyelinating disease of human brain), and exhibited antigenic similarity to SV40 (14,15). A study of PML-2 and SV40 DNAs has been carried out by Sack et al., and, as will be discussed later,our results are entirely compatible with theirs (13).

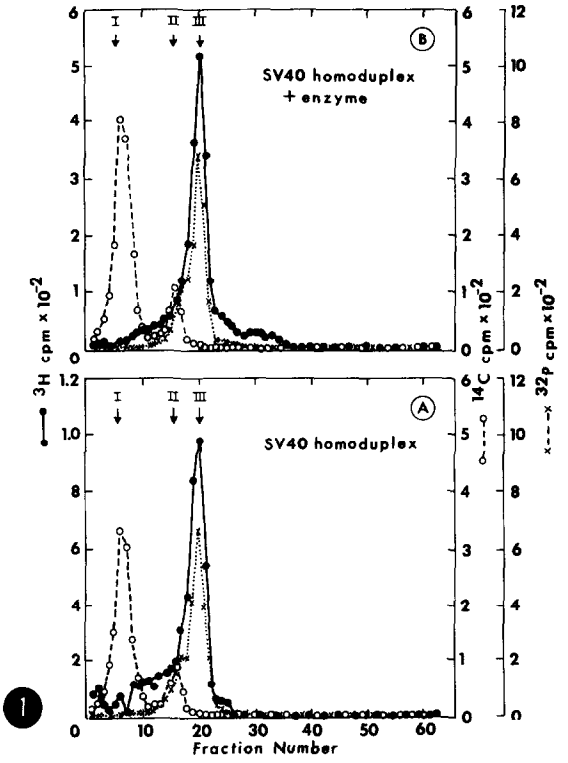
MATERIALS AND METHODS: Viral DNAs were phenol extracted from isolated virions, and purified by successive sucrose gradient and dye-density centrifugation. The ^{3}H-labeled DNAs used in the preparation of homo- and heteroduplexes were 60-90% Component I, the remaining 10-40% being Component II. Preparation of homo- and heteroduplexes could not be done directly, since the covalently closed circular DNA does not permit strand separation upon denaturation (16). The molecules were therefore first cleaved to unit length linears by treatment with the restriction endonuclease EcoRI (17,18). For heteroduplex formation, equal amounts of two different DNAs were mixed and digested with EcoRI. They were then denatured and reannealed as previously described (9,19), under conditions equivalent to atleast 15 times the $C_o t_{1/2}$ of SV40 DNA. Enyzme treatment of the DNA was in 0.1M Tris-HCL (pH8.0), o.1M NaCl and 0.01M $MgCl_2$, with 10-30 μg/ml DNA and 4 units/ml N.crassa nuclease, for 30 min at 37°C. The reactions were terminated by the addition of 0.02M EDTA. Sucrose gradient centrifugations were as in the figure legends, with solutions containing 0.01M Tris-HCL (pH8.0), 1.0M NaCl and 0.01M EDTA. Gel electrophoreses were in 0.6 x 16 cm gels of 0.5% agarose, 2% acrylamide-bis acrylamide (20:1), using the E buffer system previously described (20). After 60 min prerun, samples were electrophoresed for 15 hours at 4.5 mA constant current per gel. ^{32}P -labeled ϕX174 RFIII DNA was used as a marker in the gels.

1. Abbreviations used: SV40, Simian Vacuolating Virus 40; $C_o t_{1/2}$ is the concentration of nucleotides in moles per liter x the time needed for 50% reassociation.

RESULTS AND DISCUSSION: Homoduplexes were made from SV40 DNA alone and examined before and after enzyme treatment. Fig.1A shows the homoduplex DNA cosedimenting with the linear Component III marker (14.5s), indicating that good renaturation had taken place. The small amount of ^{3}H - labeled material sedimenting faster than 14.5s is likely due to the presence of 10-40% Component II in the starting material. This would yield shorter than unit length single strands upon denaturation of EcoRI treated linears, and some circularization and/or aggregation could occur. Fig.1B shows the homoduplexes after treatment with the N.crassa nuclease: the homoduplex again cosediments with the Component III marker. These controls yielded the same results with DAR homoduplexes. Therefore these conditions of duplex formation and enzyme treatment do not result in any fragmentation of homoduplex material. This is what one would predict, taking into account that for the N.crassa enzymes used here the relative ratio of activity with denatured versus native substrates is greater than 600 : 1 .

Homo- and heteroduplexes were then produced using a combination of SV40 and DAR DNAs (both ^{3}H - labeled). Fig.2A shows the undigested material to cosediment with native Component III. Upon enzyme treatment, two new slower sedimenting peaks, A and B , appear. Using the ^{32}P - labeled Component III marker, and the ^{14}C - labeled 21s and 16s species, the S^{20}_{w} values of peaks A and B were calculated (21), and found to correspond to molecular weights representing about two-thirds and one-third of the SV40 genome, respectively (see Table 1).

We wished to quantitate the proportion of the radioactivity present in each of the two fragment peaks, to check if they were produced in equimolar amounts, as predicted. Gel electrophoresis was employed to obtain a clear separation of the homoduplex peak and fragments A and B . A mixture of ^{3}H - labeled homo- and heteroduplexes was treated with the N.crassa nuclease preparation and was co-electrophoresed with ^{32}P - labeled ϕX174 RFIII marker. The patternof radioactivity seen in the fractionated gel is shown in Fig.3 . There is a large homoduplex peak, electrophoresing together with the marker ϕX174 RFIII, indicated by the arrow. The two fragment peaks, A and B , are well resolved. The data are summarized in Table 1 .



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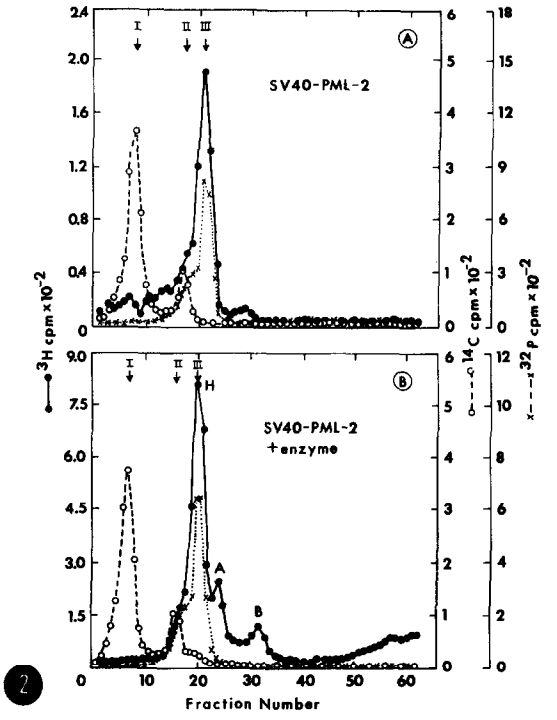

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Fig.1. Neutral sucrose gradients of SV40 homoduplex DNA before and after N.crassa nuclease treatment. 0.2ml samples were layered onto 13 ml 5-20% sucrose gradients, and centrifuged in the Beckman SW40 rotor for 15 hours at 38,000 rev/min at 10°C.Marker DNAs were added prior to centrifugation. A- before enzyme treatment. B-after enzyme treatment.(●——●) ^{3}H-labeled SV40 homoduplex DNA.(o - - - o) ^{14}C-labeled SV40 Component I and II DNA.(x . . . . x) ^{32}P-labeled SV40 Component III DNA.

Fig.2. Neutral sucrose gradients of SV40/DAR homo- and heteroduplexes, before and after enzyme treatment. Centrifugation was as for Fig.1 . A - before enzyme treatment B - after treatment with the N.crassa nuclease preparation.(●——●) ^{3}H-labeled SV40/DAR homo- and heteroduplex DNA.(o - - -o) ^{14}C-labeled SV40 Component I and II DNA.(x . . . . x) ^{32}P-labeled SV40 Component III DNA .

The molecular weights calculated on the basis of the s_w^{20} represent about 0.65 and 0.35 of the total viral genomes. The insert in Fig.3 shows the relationship evident when these molecular weights, as well as the marker RFIII, are plotted versus the relative electrophoretic mobility. The linear nature of this plot supports the accuracy of the values for the molecular weights from the gradient calculations. Further-

TABLE 1

Sucrose Gradient Analyses of Enzyme Treated SV40/DAR Homo- and Heteroduplexes

Peak	S^{20}_w	M.W. x 10^6 daltons	mean	Fraction of the genome	mean
H	14.5s	32	-	1.0	-
A	13.0-13.7s	2.0-2.4	2.2	0.6-0.7	0.65
B	10.3-10.9s	1.05-1.2	1.0	0.31-0.35	0.33

Gel Electrophoresis of Enzyme Treated SV40/DAR Homo- and Heteroduplexes

Peak	Aetual cpm	% of Total cpm	Specific Activity cpm/MW	Normalized	Fraction of (cpmA + cpmB)
H	20,190	64.1%	-	-	-
A	7.287	23.15%	3312	1.0	0.65
B	3,991	12.68%	3470	0.95	0.35

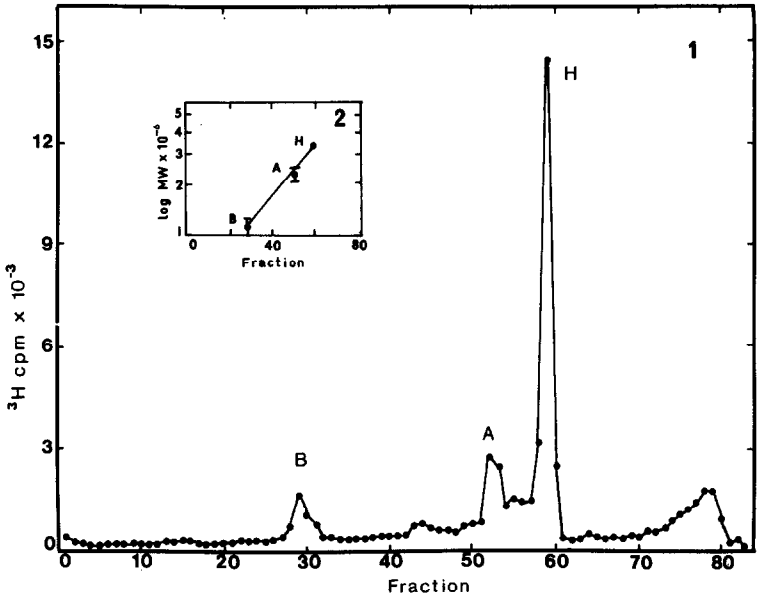

Fig.3. Agarose-acrylamide gel electrophoresis of enzyme-treated SV40/DAR homo- and heteroduplexes. A 0.2 ml sample, containing bromphenol blue, 30% sucrose, and added ^{32}P-labeled ϕX174 RFIII marker was layered onto the gel, and electrophoresed at room temperature, as described in Materials and Methods. The gel was fractionated into 2mm slices, digested with H_2O_2 and NaOH, and the radioactivity determined in 15 mls toluene-Triton X-100 scintillation fluid.H indicates the homoduplex peak, A and B the two fragment peaks. The arrow indicates the position of the ϕX174 RFIII marker DNA.

The inset is a plot of the relative electrophoretic mobilities versus the log of the molecular weights obtained from the sucrose gradient analyses. Again the arrow indicates the position of the ϕX174 RFIII DNA.

more, the amount of the radioactivity present in each of the two fragment peaks in the gel can be divided by the corresponding molecular weight. The very close agreement of these two values, as shown in Table 1 , indicates that the fragments are indeed produced in equimolar amounts, since they have the same specific activity.

Fig. 4A is a schematic representation of what is happening, illustrating the manner of production of the fragments. The region of heterology that is cleaved in these molecules can be 0.35 fractional units from the EcoRI site, in either a clockwise or an anticlockwise direction. Our initial interpretation of the data favored the location of this site in fragment Hin C of the H.influenza restriction enzyme produced fragments of SV40 (22,23) since 0.67 map units corresponds to the site of initiation of DNA synthesis (18,22) and thus the fragments we produce could well arise by cleavage at or very near this site. Recent evidence from another laboratory

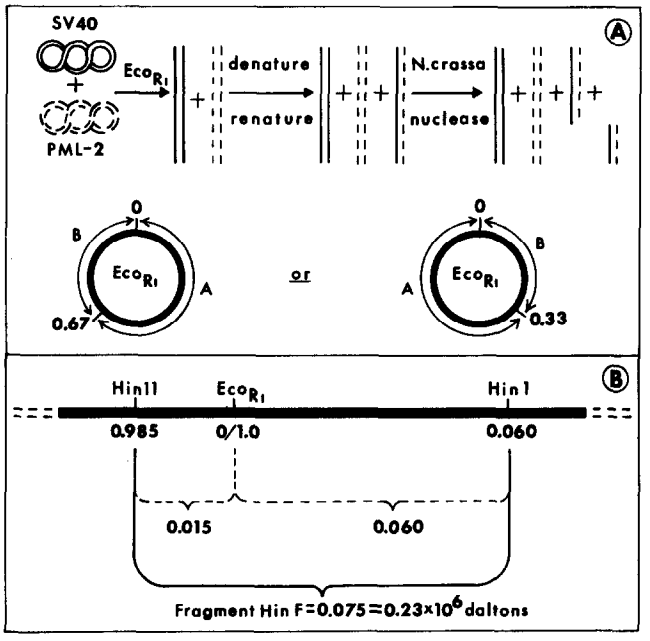

Fig.4. A:schematic illustration of the production of the fragments by enzyme treatment, showing the possible correlation of the data with the physical map of SV40. For simplicity, the Watson-Crick turns are not drawn. The solid line represents DNA of one source, SV40, while the dashed line represents DNA of another source,DAR. The two possible positions of cleavage ate indicated on the two central molecules.

B : Physical map of the SV40 genome in the region of the EcoRI cleavage site (23). Map units are fractional units of the genome from the EcoRI site,clockwise, which is designated 0 and 1.0 .

proves that this is indeed the case (13) . Comparisons of H.influenza digests of these two DNAs revealed the appearance of two new fragments, with the concomitant disappearance of fragments HinC and HinF . The authors state that their data are compatible with the presence of two small deletions in the DNA, in fragments HinC and HinF, the sum of which is at the most 0.06×10^6 daltons, or about 2% of the SV40 genome. The existence of two such small deletions is entirely compatible with our data. The cleavage by the N.crassa nuclease at a point two-thirds of the genome clockwise from the EcoRI site generates the two fragments, A and B. The small "deletion" in fragment HinF is also likely cleaved, but would result in a fragment too small to be seen under the conditions used in our experiments. Fig 4B shows that such a fragment would be at best 2-3% of the molecule, but quite possibly less than 0.1%. Needless to say, if this SV40-like virus of human origin has a divergence at or near the site of origin of DNA synthesis, a number of highly interesting possibilities are raised, especially in view of the transforming nature of SV40, and its known integration into the host chromosome during both lytic and transforming infection (24,25) . We would however like to emphasize the potential applicability of this technique for mapping mutations due to deletions or other sequence alterations. Several other single-strand specific nucleases exist that could be used in such studies, such as the S1 nuclease of Aspergillus (26) or the V1 nuclease of Vaccinia virus (27). The RNase activity of S1 is however thought to be a contaminant , and V1 is RNase free. The N.crassa endo and exonuclease exhibit near-absolute single-strand specificity with both DNA and RNA, and DNA-RNA hybrids, and this could be useful in view of the absence of restriction nucleases for RNA. Exploitation of the above techniques for mapping of mutants of such viruses as the double-stranded RNA containing Reovirus is therefore feasible.

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