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DNA orientation using specific avidin-ferritin biotin end labelling

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ABSTRACT

The orientation of DNA molecules has been determined by labelling one of the molecule end with a Biotin-labelled analog of dTTP (Bio-dUTP) and then by complexing the Bio-dUTP with Avidin-Ferritin. DNAs of ØX174, pBR322 and SV40 were end labelled with Bio-dUTP and imaged by Electron Microscopy (EM). This is a rapid, general method to unambiguously determine the orientation of DNA molecules for precise mapping and quantification of DNA secondary structures or protein-DNA interaction sites using EM.

INTRODUCTION

The biochemical study of nucleic acids often requires the mapping of important structural regions such as promoters, operators, introns, exons and the actual gene. Radioactive end-labelling of fragments containing such regions provides a means of detecting low concentrations of the fragment when studied using enzymatic or chemical probes. But in electron microscopy, the ends of such labelled fragments are indistinguishable. Therefore, to determine the orientation of the DNA fragment, a variety of restriction digestions followed by computer analysis of the electron micrographs is required to determine the orientation of the original molecule (1,2). Thus, until now distinguishing the ends of nucleic acids to orient the molecule has been a complicated process. We have significantly simplified this procedure by labelling only one end of a double-stranded DNA molecule with biotinylated dUTP (Bio-dUTP).

Recently, biotinylated dNTPs have been successfully substituted for radiolabelled dNTPs (3). The method of imaging the biotinylated residues requires the reaction of Avidin with the biotin moiety. This reaction of Avidin with the Biotin is detected easily by colorimetric, immunofluorometric methods (4,5) and has particularly low detection limits as a result of the high specific affinity constant of the reaction ($Kd=10^{-15}$) (6). In some cases, the detection sensitivity approaches the sensitivity

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obtained when using radioactive compounds (7). In fact, there are literature reports which detail the use of biotinylated nucleic acids for such purposes as indicators in hybridization and sequencing (8,9,10).

When Avidin is coupled to ferritin, an electron-dense iron metalloprotein, the complex is visible using EM. Several recent studies have taken advantage of the EM visibility of the coupled Avidin-ferritin (11,12). The Avidin-ferritin complex has already been used to reveal the site of DNA repair in ultraviolet irradiated cells by incorporating Bio-dUTP at repair sites (11). The repair sites which incorporated the Bio-dUTP were then visualized by complexation with the Avidin-ferritin moiety. In another study, single-stranded regions in Cauliflower Mosaic Virus (12) were imaged using EM following incorporation of Bio-dUTP by Klenow fragment polymerase (Kf polymerase). Bio-dUTP has also been incorporated by Kf polymerase at the 3' OH end of a synthesized oligonucleotide probe (13). However such labelling was possible only because the probe contains an adenine at one of its terminal single strand ends.

In this report, we show that by judicious choice of restriction enzymes, it is possible to label a single end of a double-stranded DNA and thereby use EM to determine its orientation in a single step. Moreover this method is also used to identify a specific restriction fragment in a mixture of fragments. Through this method, linearized DNAs of a bacterial phage, a plasmid, and a eucaryotic virus were oriented using darkfield electron microscopy without the use of cytochrome C (14,15).

MATERIALS AND METHODS

- DNAs : ØX174 Replicative Form RFI (5386 bp), pBR322 (4363 bp), and SV40 (5343 bp) were purchased from New England Biolabs. In this work ØX174 DNA always refers to double stranded DNA.

- Enzymes : restriction enzymes were purchased from Boehringer-Mannheim, New England Biolabs, or Genofit. Kf DNA polymerase was purchased from BRL. All enzymes were used without further purification.

- Nucleotides : all deoxyribonucleotides were purchased from Boehringer-Mannheim.

- Biotinylation reagents : the Bio-dUTP and monomeric Avidin-ferritin were gifts (G. de Murcia).

- FPLC : following restriction and labelling, the DNAs were purified using a Pharmacia FPLC P-500 system with a 0.5 x 5 cm Superose 6 column operating at a flow rate of 50 μl/min.

Preparation of DNA with a bio-dUTP label

The DNAs were restricted under standard buffer conditions (10 mM Tris/HCl pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$) in a 30 μl solution (DNA concentration 30 μg/ml) with 5.0 unit of enzyme. The digests were incubated for 1 h at 37° C.2 μl of a 100 μM Bio-dUTP solution was added along with any necessary dXTPs (1 μl of a 1 mM solution) and 0.6 unit of Kf polymerase. The volume of the reaction was adjusted to 50 μl by the addition of 100 mM NaCl, 10 mM Tris-HCl, 0.2 mM EDTA, pH 7.4 (Buffer A) and incubated at 37°C for 45 min. Unincorporated Bio-dUTP and dXTPs were removed by FPLC. Buffer A was used because it prevents non-specific fixation of ferritin to DNA (12). DNA fractions of 25 μl were pooled and adjusted to 5 μg/ml.

Avidin-ferritin labelling

To 25 μl of the purified biotinylated DNA, 2 μl of Avidin-ferritin (62 μg/ml) were added to yield a final molar ratio of 10/1 Avidin-ferritin/Biotin. The reaction was incubated for 1 h at room temperature. A second restriction was then performed by adding 2 μl of a 50 mM $MgCl_2$ solution and 5 unit of restriction enzyme. The DNA was then purified using FPLC to remove the restriction enzyme and unfixed Avidin-ferritin.

Electron microscopy

After each reaction step, aliquots were removed and electron micrographs were obtained.

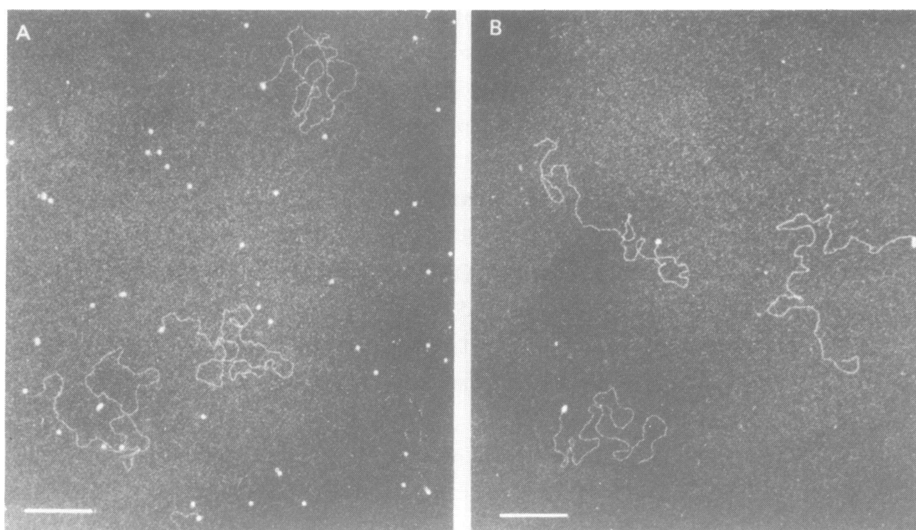
The DNA (5 μl of a 0.5 μg/ml solution) was deposited on a carbon-coated 600 mesh grid activated by glow discharge in the presence of pentylamine (16). Grids were stained with a 2 % uranyl acetate-solution, dried and observed by darkfield electron microscopy in a Zeiss EM 902 microscope.

In order to verify that the lengths of the different molecules showing ferritins were in agreement with the theoretical values, prints at 150,000 x magnification were digitized using an ACT digitizer and processed with in house software on a KONTRON microcomputer.

Aim of the method

When Type II restriction endonucleases cleave DNA, one of 3 different types of ends is generated, depending on the enzyme (17). These different types of ends are : 1) 3' recessed ends such as generated by <u>EcoRI</u> cleavage, 2) 5' recessed ends such as generated by <u>PstI</u> cleavage, and 3) blunt-ends such as generated by <u>PvuII</u>. Only 3' recessed ends are good substrates for the Kf polymerase activity.

In order to obtain DNA with a Bio-dUTP label at only one of the ends, there are two simple methods. In the most simple case, it is possible to fill



Panel I. Figure A : The terminal Bio-dUTP incorporated in the restriction site is revealed by Avidin-ferritin complexation for ØX174 DNA molecules linearized by AvaII. Background ferritins are observed before passage on the Superose 6 column. Two of the three DNA molecules presented are end labelled with one ferritin, whereas the other at the top of the picture is end labelled with two ferritins.
Figure B : Biotinylated ØX174 DNA molecules end labelled with Avidin-ferritin are presented after passage on the Superose 6 column. Unfixed ferritin have been removed and are not observed. Two of the DNA molecules shown are end labelled with two ferritins, the other with one ferritin.
The bars equal 0.2 μm.

in one end of the DNA using Kf polymerase and Bio-dUTP and not incorporate Bio-dUTP at the other end of the molecule. In this case only one adenine is involved in the restriction sequence. This is demonstrated in this study for the AvaII digest of ØX174 RF I DNA, the AvaI digest of pBR322 DNA, and the AccI digest of SV40 DNA. The other method to obtain DNA labelled with Bio-dUTP at one end only requires that there is two adenines involved in the restriction site. Initially, both ends are labelled with Bio-dUTP, followed by cleavage with a second enzyme. The second digest creates two labelled fragments, and possibly other unlabelled fragments. By carefuly choosing the second enzyme, the two labelled fragments differ in size significantly and are resolvable by EM. This method is illustrated in this study for the BamHI and PvuII digests of pBR322 DNA. This method of labelling is also useful to identify particular restriction fragments in a mixture, as indicated for the AccI digest of ØX174 DNA.

Table I : Terminal incorporation of bio-dUTP in ØX174, pBR322, SV40 DNAs linearized respectively by AvaII, AvaI and AccI. AvaI and AccI.

DNA	+	ENZYME	DOUBLE LABEL	SINGLE LABEL	INTRAMOLECULAR LABEL	UNLABELLED
ØX174	+	AvaII	3 %	59 %	11 %	27 %
pBR322	+	AvaI	2 %	65 %	29 %	4 %
SV40	+	AccI	3 %	54 %	11 %	32 %

The percentage of each type of observed ferritin labelled molecules is presented. For the three DNAs tested, more than 50 % the molecules are terminally biotinylated. The double labelling is low, less than 5 %, and the 10 % intramolecularly labelled molecules observed for ØX174 DNA and SV40 DNA are the 5 % and 15 % molecules initially nicked before restriction of the supercoiled DNAs. For pBR322 DNA the percentage of intramolecularly labelled molecules is high as the starting solution contains 20 % of nicked molecules and as the enzyme AvaI introduced new nicks.

RESULTS

For each of the following analyses 300 molecules were observed at a magnification of 50,000 x (200,000 x on the video screen of the microscope). Starting DNA solutions were supercoiled to a level of 95 % for ØX174 DNA, 79 % for pBR322 DNA and 85 % for SV40 DNA. Remaining molecules were mainly relaxed with a small amount of linear DNA (less than 2%).

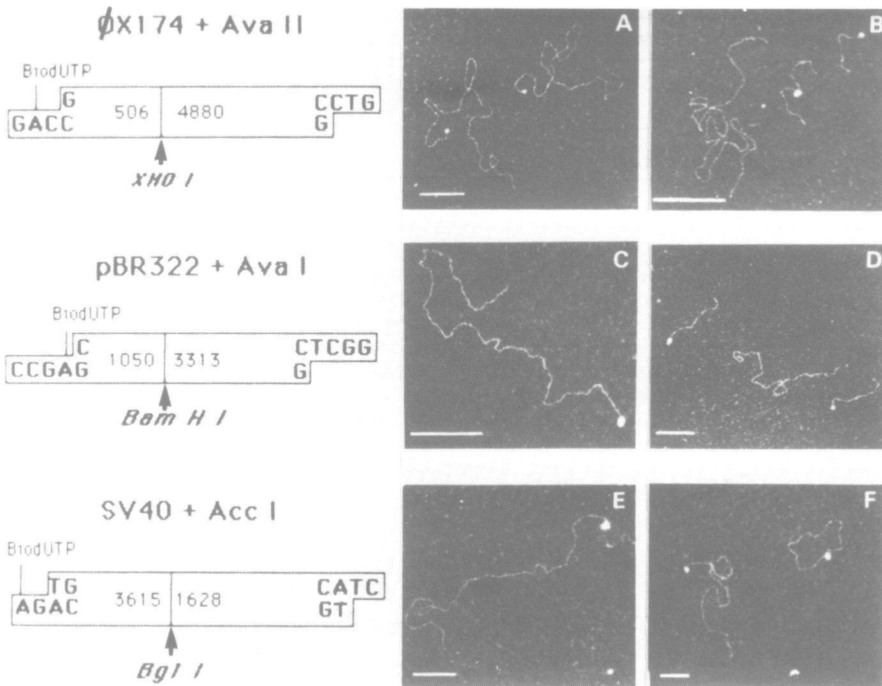
After incubation with AvaII and AccI respectively, ØX174 DNA and SV40 DNA were 100 % linearized. 89 % of pBR322 molecules were linearized by AvaI, the others being only nicked.

When DNAs were observed after Bio-dUTP incorporation and purification, their general structural features were unaffected.

Avidin-ferritin end labelled ØX174 DNA molecules are presented in panel I figure A before FPLC purification and figure B after purification using the Superose 6 column which reduces the background of unbound avidin-ferritin.

Selective labelling of a single end of double-stranded DNA molecules

Results are presented in table I and panel II. As revealed by avidin-ferritin complexation and EM observation, 3' recessive ends are labelled with Bio-dUTP. To perform this labelling however it is necessary to include dXTPS in the reaction mixture if there are other bases between the end of the double-strand and the adenime in the restriction site. dGTP and dCTP respectively were added to ØX174 DNA restricted by AvaII and SV40 DNA restricted by AccI, whereas no other dXTPs were needed for pBR322 DNA



Panel II : Selective labelling of one end of a DNA molecule : Diagrams on the left are models of the three specifically end labelled molecules. Supercoiled ØX174, pBR322 and SV40 DNAs are cut respectively by AvaII, AvaI and AccI. Bio-dUTP is then incorporated in the adenine containing end by Klenow fragment DNA polymerase in presence of the necessary dXTP. Avidin ferritin is added to the biotinylated DNA. To check the location of the labelling, a second restriction is performed. In the examples presented the labelled DNAs were respectively cut by XhoI, BamHI and BglI. The lengths of obtained fragments are given in base pairs.

In figure A two entire ØX174 DNA molecules labelled with ferritin at one end are observed. In figure B the labelled end is established after restriction with XhoI. The small fragment is labelled whereas the large one is not. On figure C pBR322 DNA cut by AvaI labelled at one end with ferritin is presented. The DNA solution after restriction by BamHI contains only small labelled fragments as presented in figure D. In figure E supercoiled SV40 DNA end labelled with ferritin after restriction by AccI is shown. In figure F an entire molecule labelled at one end (left part of the picture) and the large fragment (upper right of the picture) obtained after restriction by BglI are shown. This last one appears circular due to a close location of the two ends.

For all figures the bar equals 0.2μm.

restricted by AvaI as indicated in the diagrams of panel II. For the three DNAs, most of the population of the molecules is terminally labelled. Figures A, C, E show examples of ØX174 DNA, pBR322 DNA and SV40 DNA labelled with

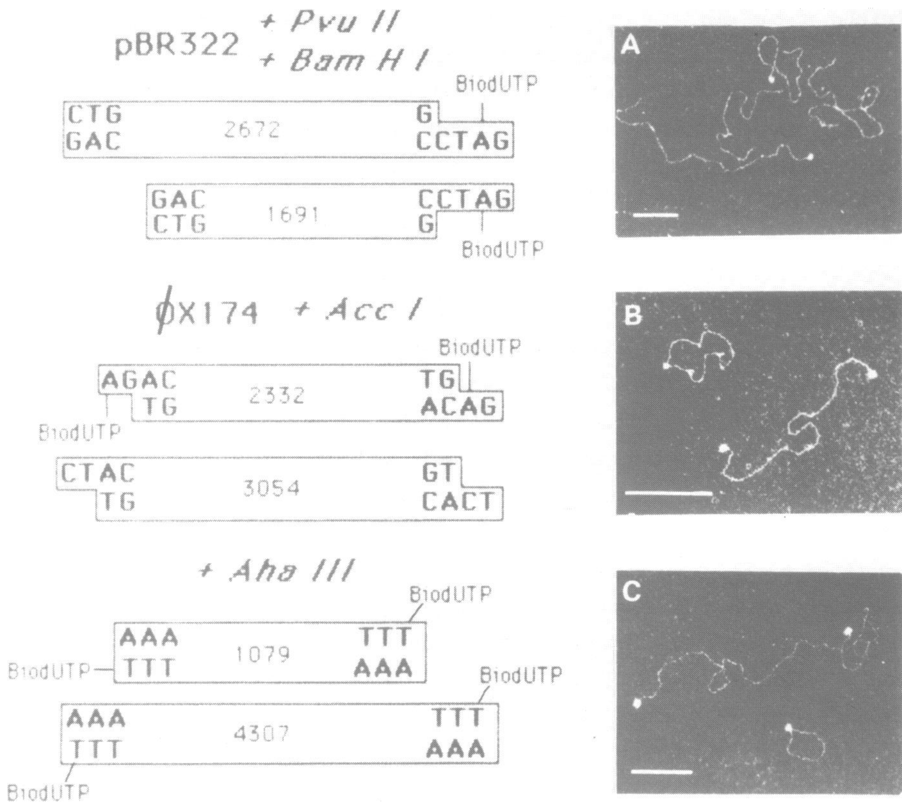
ferritin at one end. The efficiency of the labelling is indicated in table I. 50 % of the molecules are at least singly labelled and less than 5 % are labelled at both ends. In most cases, only one ferritin is observed on the molecules but sometimes, due to the natural tendency of ferritin to aggregate, protein dimers have reacted with the biotinylated end. A significant number of molecules are also intramolecularly labelled (10 - 30 %). For ØX174 DNA and SV40 DNA, the percentage of such labelled molecules is close to that of molecules initially nicked which suggests that the Kf polymerase has incorporated Bio-dUTP at nicks. For pBR322 DNA this percentage is high as we have observed that AvaI contains some nicking activity and as 21 % of the starting DNA solution was already nicked.

To check the orientation of the DNA molecules, we digested the different Avidin-ferritin labelled DNAs with another restriction enzyme which has only one site on each of the three DNAs. The two obtained fragments have different lengths as indicated in the diagrams of panel II and are easily identified by EM. Only small labelled fragments are observed when a second restriction is performed on ØX174 DNA or on pBR322 DNA respectively by XhoI and BamHI. These small labelled fragments are showned in figures B and D of panel II. When labelled SV40 DNA is cut by BglI, the large fragment is labelled whereas the small one is free of Avidin-ferritin (fig F in panel II). These results confirm that the Bio-dUTP is effectively incorporated in the adenine containing end. The orientation of these three DNA molecules is obtained in a one step restriction reaction and the integrity of the whole molecule is maintained by this procedure.

Labelling both ends of a double-stranded DNA molecule

When circular pBR322 DNA is cleaved by BamHI (single restriction site at position 375), both single stranded 3' recessive ends contain an adenine (5' G/GATCC 3'). Bio-dUTP was so incorporated at both ends by Kf polymerase in these linearized molecules in presence of dATP and dGTP. After purification the DNA molecules were complexed with Avidin-ferritin and cut by PvuII at the site 2066. The unfixed Avidin-ferritin and enzymes were removed by FPLC and the DNAs were observed. A model of the biotinylated fragments obtained is shown in the diagram A of panel III.

73 % of the total molecules observed were fragments labelled at only one end. Two of them, a small and a large one, are presented in picture A of panel III. The other molecules were unlabelled ones and few showed intramolecular Avidin-ferritin labelling. By this two step restriction method the pBR322 molecules were also oriented, but in this case, the integrity of



Panel III : Double terminal labelling of DNAs : Diagrams on the left present a summary of this procedure applied to ØX174 and pBR322 DNAs. The first diagram presents an example of pBR322 DNA cleaved by PvuII and BamHI and terminally labelled at the BamHI site. In figure A the obtained small and large labelled fragments are observed with two unlabelled ones.

For ØX174 DNA AccI produces two fragments with 3' recessive ends. One of them is labelled with Bio-dUTP at both ends. Two labelled fragments are shown in figure B. One of them is a linear one whereas the second is circular due to interaction between the two terminal ferritins. When ØX174 DNA is cut with AhaIII the two obtained blunt end fragments can be labelled. Figure C shows the small circularized fragment and an entire labelled ØX174 DNA molecule which is cleaved at one site.

For all figures the bar equals 0.2 μm.

the molecules is not maintained. This method is similar to the method used to isolate fragments for chemical sequencing (18).

Double terminal labelling was also visualized on ØX174 RF DNA restriction fragments. ØX174 RFI DNA was restricted by AccI which produces two fragments : 2332 bp and a 3054 bp. The small fragment contains adenine at each

of its ends whereas the large fragment contains the corresponding thymidine as presented in the diagram B of panel III. These fragments were biotinylated, complexed with Avidin-ferritin and analysed by EM.

No large labelling fragments were observed but only small ones. The population of Avidin-ferritin labelled fragment consisted of three types. 17 % were labelled at one end and 37 % were labelled at both ends. Within those 37 %, 5 % were linear and 32 % were circular. The natural tendency of ferritin to make dimers may explain this circularization. These fragments are shown in figure B of panel III.

Labelling of other types of restriction fragments

Kf polymerase is able to exchange the last 3' OH nucleotide of a blunt-end (19). To check this exchange we attempted to incorporate Bio-dUTP at the ends of ØX174 DNA cut by AhaIII which has two restriction sites (5' TTT/AAA 3') at position 327 and 1406. The two obtained blunt-end fragments containing a terminal 3' OH thymidine are presented in the diagram C of panel III. These fragments were biotinylated, deposited on grids and observed. The labelling was efficient on both small and large fragments. They presented one (30 %) or two (26 %) labelled ends. Most of these doubly labelled fragments were circularised as observed for ØX174 DNA cut by AccI, but higher yield of circularisation was observed for the small fragments than for the large ones. Figure C of panel III presents a small circular double-labelled fragment and an entire linearized ØX174 DNA molecule labelled with two ferritins at both ends.

Other types of blunt-ends were also tested. When thymidine was in 5' phosphate position, as for ØX174 DNA fragments obtained by restriction with XmnI it was not efficiently replaced by Bio-dUTP, 8 % of labelling. When there is no terminal thymidine at the end of a blunt-end fragment as for pBR322 DNA restricted by PvuII, no labelling was observed.

5' recessive ends obtained by restriction of pBR322 by AatII were not significantly labelled with Bio-dUTP (8 %). No 3' ⟶ 5' polymerase activity is known for Kf polymerase and this low incorporation may be explained by the fact that adenine is the first unpaired nucleotide of the produced single strand end (5' GACGT/C 3').

DISCUSSION

Enzymatic biotinylation of DNA by nick translation is used for the synthesis of nucleic acid probes (3) but terminal biotin labelling is not as common. We have shown in this paper that biotinylation can be performed on a

chosen end of a DNA molecule. By the biotinylation method that we have developed, orientation of DNA is obtained in less than 4 hours. Moreover, as opposed to other ways of DNA orientation, the proposed method maintains the integrity of the molecule. Such labelling was possible for the DNAs studied because all have a single assymmetric restriction site containing one adenine. For other circular DNAs which do not have such sites it is possible to perform the orientation by labelling both ends of the linearized molecules and then cut a second time with another restriction enzyme. In this case the integrity of the whole DNA molecule is not maintained.

Due to the high affinity of Avidin-ferritin for Biotin and to the not stringent conditions used in these experiments there was no need of fixative agents such as glutaraldehyde. The electron microscopic observation done in this work yields precise information on the efficiency of labelling and location of the labelled sites.

We have shown that more than 54 % of the DNA molecules have incorporated Bio-dUTP at one of their extremities. As it was revealed by DNA-Avidin-ferritin complexes observation, the best yield is obtained with pBR322 DNA. This result is correlated with the fact that Bio-dUTP is the first nucleotide to be incorporated whereas for ØX174 DNA and SV40 DNA the adenine is in second position (Diagrams of the panel II). The biotinylation yields obtained by our method seem lower compared to the 80 % of avidin-ferritin bound to the Cauliflower Mosaic Virus single strands (12). However it must be kept in mind that natural single strand discontinuities present in this DNA are longer (10,21 and 17 bases) than the maximum single stranded regions of four bases produced by restriction enzymes. Moreover these single-stranded structure contain up to 10 adenines allowing a higher binding of Avidin-ferritin. By using Kf polymerase for Bio-dUTP incorporation into a DNA probe, Murasugi and Wallace obtained a maximum yield of 30 % (13). The incorporation that we have noticed in nicks is most probably due to a contaminating residual 5' ⟶ 3' exonuclease activity of the Kf polymerase which has preceded the 5' ⟶ 3' polymerase activity. Some 3' ⟶ 5' exonuclease activity might explain the small amount of double labelling observed (see table 1).

According to our results, it is possible to label one or both ends produced by other available restriction enzymes. Enzymes giving 3' recessive termini with only one adenine involved in the restriction site such as BstNI (5' CC/AGG 3') and those giving blunt-end with 3'OH terminal thymidine at only one end can be used to efficiently end label DNA with Bio-dUTP in a single

step restriction reaction. Enzymes giving 3' recessive ends with an adenine on both produced termini or those giving blunt ends with a terminal 3'OH thymidine at both ends can be also used to label DNA. In this case the orientation is performed with a second restriction of the doubly labelled molecule.

As Kf polymerase is unable to incorporate nucleotides at 5' recessive termini, other polymerases must be used to label such extremities. T4 DNA polymerase, which has a higher 3' ⟶ 5' exonuclease activity than that of Kf polymerase, is able to digest the 3' protruding extremity and to exchange the last 3' OH base (20) with a Bio-dUTP if this one is a thymidine. Terminal transferase which is able to add a dXTP at a 3'OH protruding extremity in presence of Mg^{2+} or to blunt-end in presence of Co^{2+} can also be used to label these types of ends (21).

Biotin labelling can be performed through the use of other biotinylated deoxyribonucleotides. In this work, we have used Bio-dUTP to label one end of DNA molecules, but as Bio-dATP is also available, the other end of the molecules could be labelled with the same protocol. The use of bio-dGTP or Bio-dCTP, not yet synthesized to our knowledge, will widen the field of application of this method.

Proteins with high affinity for particular DNA sequences such as RNA polymerases, repressors or Z-DNA antibodies could also be used to orient the DNA. However, all the studied DNA molecules do not contain these particular sequences or structures.

The essential goal of the terminal biotinylation procedure that we present is to obtain orientated DNA molecules. Orientation facilitates fine mapping of DNA-protein binding sites studied by EM. Moreover, it will allow comparisons of the binding frequency of these sites and will establish a clear hierarchy of protein affinities for these differents sites. In addition, particular secondary structures could not only be mapped, but the ability of DNA sequences to adopt such particular structures could also be compared.

In conclusion, through the use of the Biotin-Avidin system an efficient protocol to determine DNA orientation is presented. The major advantage of the biotinylation procedure we presented resides in the various combinations offered to perform it and in the possibility to label a chosen end of the DNA molecule.

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