HYBRIDIZATION CHARACTERISTICS OF ENZYMATICALLY SYNTHESISED DNA COMPLEMENTARY TO MOUSE IMMUNOGLOBULIN MESSENGER RNA

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1. Introduction

It is not known what degree of diversity occurs in the DNA sequences coding for the variable (V) and the constant (C) regions of immunoglobulin molecules. A direct test of the number of genes present in the germ-line may be made by molecular hybridization studies between a purified immunoglobulin mRNA and nuclear DNA. The mRNA for the mouse immunoglobulin light chain (L-chain) has now been partially purified from a number of myelomas [1–5], and an L-chain mRNA fraction, from P3K tissue culture cells, has been used in preliminary hybridization studies [6]. These studies showed that the L-chain mRNA fraction (which is about 25–50% pure L-chain mRNA [7]) hybridizes to nuclear DNA with biphasic kinetics, consistent with the presence of components which hybridize with repetitive and with non-repetitive elements in the DNA. The role of V- or C-genes in these hybridization experiments was not clear, however, since control experiments showed that non-immunoglobulin mRNA species hybridize similarly to the L-chain mRNA fraction.

In order to obtain information on the position of the repetitive and non-repetitive elements in the mRNA, complementary DNA copies (cDNA) of the L-chain mRNA fraction were prepared using the RNA-dependent DNA polymerase (reverse transcriptase) of avian myeloblastosis virus. Previous studies with this enzyme showed that L-chain mRNA could be copied into DNA in a transcription reaction which starts at the poly(A) region of the mRNA (3′-terminal region) [8–10]: such cDNA was estimated to be on average about 100,000 daltons in molecular weight. The 3′-terminal region of the mRNA is known to be complementary to the carboxy terminal region of the L-chain protein (the C-region) and cDNA of 100 000 molecular weight could contain at best only coding sequences for the C-region and none of the V-region of the L-chain.

The results described in the present studies show that reverse transcriptase cDNA preparations, made from the P3K L-chain mRNA fraction (the LE mRNA fraction), hybridize to excess nuclear DNA with a single sharp transition corresponding to genes in the unique fraction of the DNA (estimated to be 2–3 copies per haploid genome). The contribution to the hybridization profile of L-chain C-region coding sequences and untranslated sequences is discussed.

2. Materials and methods

Mouse P3K L-chain mRNA fraction (previously designated the LE mRNA fraction [6]) was prepared as described [7]. P3K cells are a tissue culture line derived from mouse MOPC-21 plasmacytoma cells [11].

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Avian myeloblastosis virus was obtained from the Microbiological Research Establishment, Porton Down, Wilts., and the RNA-dependent DNA polymerase was prepared by the method of Kacian et al. [12]. ^{32}P-labelled cDNA was prepared using α-[^{32}P]deoxyguanosine triphosphate (New England Nuclear, Boston, Mass., USA; specific activity 100 Ci/mmole; 10 μCi per incubation) in 50 μl volume containing 0.5 μg LE mRNA, 10 μl reverse transcriptase enzyme, 1 μg oligothymidylic acid (p T_{10}, P.L. Biochemicals, USA), 50 mM Tris–HCl, pH 8.3, 20 mM dithiothreitol, 6 mM $MgCl_2$, 60 mM NaCl, 1 mM d ATP, d TTP and d CTP and 50 μg/ml actinomycin D (Sigma Chemical Co. Ltd., Surrey, England). The mixture was incubated for 1.5 hr at 37°C then diluted with 1 ml H_2O. The mixture was boiled for 2 min, chilled, treated with 10 μg of pancreatic ribonuclease (previously boiled for 5 min to inactivate contaminating deoxyribonuclease) for 2 min at 37°C and then extracted with 1 volume of phenol–chloroform after adding $^1/_{10}$ volume of 1 M Tris- HCl pH 9. After centrifugation to separate the aqueous from the phenol layer, the phenol- chloroform layer was re-extracted twice with fresh aqueous and the cDNA was precipitated from the pooled aqueous material with 2 vol. of alcohol (after the addition of 50 μg *E. coli* tRNA). The triphosphates were removed from the cDNA by passage through a Sephadex SP-50 column in 0.3 M NaCl, 10 mM sodium acetate pH 5.0, 0.5% SLS.

E. coli DNA polymerase DNA transcripts of the LE mRNA were prepared by the method described previously [13].

Nuclear DNA was prepared from MOPC-21 solid tumours (maintained in BALB/c mice) basically as described [14] with modification as follows. The ribonuclease digestion of the deproteinised DNA was carried out in 15 mM NaCl, 1.5 mM sodium citrate pH 7 by the addition of pancreatic ribonuclease to 200 μg/ml and T_1 ribonuclease to 1 unit/ml. Incubation was for 2 hr at 37°C followed by 1 hr at 37°C in the presence of 0.1% SDS and 0.4 mg/ml pronase. Shearing of the DNA was by sonication.

Estimation of nuclear DNA renaturation was conducted optically as described previously [14] and cDNA- DNA hybridizations were carried out at 70°C in 0.24 M sodium phosphate buffer pH 6.8. Aliquots were removed at various times and fractionated on 1 g hydroxyapatite (Biorad, Bio-gel HTP) as described [15].

3. Results and discussion

The cDNA made from the LE mRNA fraction represents transcripts beginning at the poly(A) (the 3′-end) of the mRNA [8–10]. Since transcription is initiated at this defined position on the mRNA, an estimate of the cDNA molecular weight will determine the proportion of the mRNA which has been copied. In this case, a molecular weight determination might also allow an estimate of the amount of C- and V-region coding sequences which have been copied into cDNA. Fig. 1 shows alkaline sucrose gradients of cDNA made with reverse transcriptase and with *E. coli* DNA polymerase. The latter product has a broad size distribution with an average molecular weight of around 23 000 (N. Proudfoot, unpublished results): in comparison to this material, the reverse transcriptase cDNA product sediments in a position consistent with an average size of between 300–350 nucleotides [16]. The reverse transcriptase cDNA size range, however, is heterogeneous with a proportion (around 10–20% being smaller than 300 nucleotides)

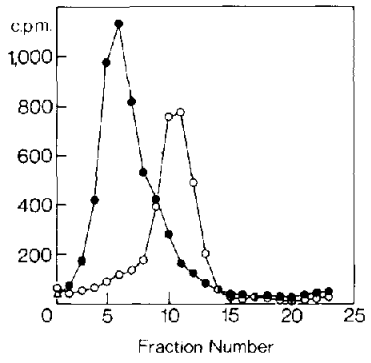

Fig. 1. Alkaline sucrose density gradient centrifugation of [^{32}P]cDNA. 10^{-4} μg of [^{32}P]cDNA, prepared with reverse transcriptase, and 10^{-4} μg [^{32}P]cDNA, prepared with *E. coli* DNA polymerase, were sedimented in separate 5- 20% sucrose gradients containing 0.1 N NaOH and 0.5% SDS. Centrifugation was carried out in an SW 6 × 14 ml rotor for 24 hr at 25°C and 38 000 rpm on a MSE 65 centrifuge. Fractions (0.5 ml) were taken for scintillation counting. Sedimentation is left to right. (●——●) DNA polymerase product; (○—— ○) reverse transcriptase product.

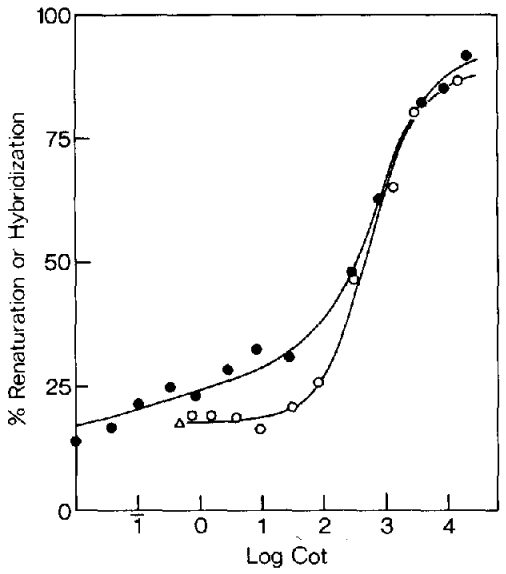

Fig. 2. Renaturation of MOPC-21 tumour DNA and hybridization with [^{32}P]cDNA. Renaturation: Two aliquots of DNA (10 mg/ml and 0.1 mg/ml) were boiled for 5 min in 0.012 M PB and then reannealed at 70°C in 0.24 M PB. Samples were withdrawn at various times and assayed optically for increase in duplex content. Hybridization: A mixture of 5 mg MOPC-21 tumour DNA and 10^{-4} μg [^{32}P]cDNA (prepared with reverse transcriptase) were boiled in 1 ml 0.02 M PB and annealed as above, but samples were analysed by hydroxyapatite fractionation. (●——●) MOPC-21 DNA renaturation; (○——○) cDNA hybridization; (△) cDNA zero time binding to hydroxyapatite. Co = Initial DNA concentration (mol. nucleotide litre^{-1}); t = time in seconds; PB = sodium phosphate buffer pH 6.8.

and only a small amount (less than 5%) being larger than 400 nucleotides.

Fig. 2 shows both the optical renaturation profile of MOPC-21 myeloma nuclear DNA and the hybridization of reverse transcriptase cDNA to the nuclear DNA. The renaturation profile of the nuclear DNA shows that roughly 30% of the sequences renature rapidly: these sequences consist of the highly repetitive satellite DNA and sequences of intermediate repetitive nature [17]. The major phase of renaturation (about 70%) occurs in a final transition (with $Cot^{d}_{½}$ of about 1000) corresponding to the unique sequences (reiterated only a very few times). The hybridization of the cDNA with myeloma DNA appears, in contrast to the range of sequence repetitions in the nuclear DNA, to occur in a *single*, sharp transition indicating that *all* the sequences present in the cDNA are hybridizing with the same rate to the nuclear DNA (i.e. to genes which are repeated about the same number of times). The $Cot^{d}_{½}$ of the transition is around 630 which represents sequences which are reiterated approximately 2–3 times in the haploid genome. The single hybridization transition of the reverse transcriptase cDNA contrasts with the biphasic pattern described for the mRNA hybridization [6]. Thus the element in the mRNA which hybridizes to the repetitive DNA does not reside at the 3′-terminus of the mRNA. Furthermore, in the previous experiments [6] the contribution of the L-chain specific mRNA sequences to the unique sequence transition was uncertain due to the inability to achieve vast DNA excess conditions. In the present experiments, there can be little doubt that the L-chain specific cDNA is contributing to the hybridization of the cDNA since the transition consistently reaches 85–90% completion (the apparent inability of the cDNA to hybridize to 100% is probably partly due to the presence of a proportion of cDNA molecules of insufficient size to form a stable hybrid under the conditions used).

The results demonstrate, therefore, that cDNA (with an average size of 300–350 nucleotides) hybridizes to unique (non-repetitive) sequences in the genome. Although the cDNA copy is made from the 3′-end of the mRNA, there is still uncertainty about the contribution of the L-chain C-region *coding* sequences to the cDNA hybridization since it appears that the L-chain mRNA contains about 350 bases which are untranslated [7]. Although it is likely that these bases are distributed in some way between the 5′- and the 3′-termini of the mRNA, it is possible that the majority of the untranslated bases occur at the 3′-end in which case a substantial proportion of the cDNA population will consist of copies of this 3′-untranslated sequence. Furthermore, it is likely that a stretch of poly (dT) (transcribed from the poly(A) of the mRNA) occurs at the 5′-terminus of the cDNA [18]. In view of these possibilities and the fact that the LE mRNA fraction is not pure L-chain mRNA, the cDNA population studied here may well contain very limited amounts of C-region coding sequence and most of the observed hybridization may be due to the 3′-untranslated sequences. Experiments are in

progress to determine the length of the 3′-untranslated sequence.

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