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Cold in-situ-hybridization of human T-cell leukemia virus type 1: Production of biotinylated probes by polymerase chain reaction and improvement of procedures

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Summary: In order to detect histochemically signals of human T-cell leukemia virus type 1 (HTLV-1) proviral DNA and its proviral DNA in MT-2 cells, we made highly biotinylated probes corresponding to parts of HTLV-1 proviral DNA by means of 2 times polymerase chain reaction (PCR) and improved the procedures of DNA-RNA and DNA-DNA cold in-situ-hybridization (cISH) for HTLV-1. We produced 7 highly biotinylated double-stranded DNA probes for the long terminal repeat, gag-pol region, env region and pX tax and rex regions of HTLV-1. DNA-RNA cISH of these probes was established, evaluating the effect of 17% polyvinyl alcohol solvent for reducing non-specific alkaline phosphatase reaction in the process of visualizing hybridized probe and suggesting the necessary prehybridization incubation with hybridization buffer containing salmon DNA for diminishing non-specific binding of probes with tissue. On the other hand, DNA-DNA cISH of a cocktail of these 7 probes could show only too faint dot-like stain in nuclei to be applied to a study of HTLV-l-related lesions, denaturing DNA in sections and probes in aluminum box for 10 min at 200℃, incubating the sections with probes for hybridization for 3 days at 45℃ and performing overnight alkaline phosphatase reaction by using 17 % polyvinyl alcohol solvent after streptavidine-biotinylated alkaline phosphatase system to detect the hybridized probes.

Key words: HTLV-1, DNA-DNA cold in-situ-hybridization, DNA-RNA cold-in-situ-hybridization, MT-2, biotinylated double-stranded DNA probes, polymerase chain reaction

Introduction

We detected signals of human T-cell leukemia virus type 1 (HTLV1) proviral DNA pX Tax region in human malignant lymphoma cells by means of cold in-situ-hybridization (cISH) employing a highly biotinylated concatamer probe synthesized by polymerase chain reaction (PCR) (Hasui et a1. 1994). The cISH employing the concatamer probe could present stronger signals than the cISH employing a 159bp-long probe for HTLV-1 proviral DNA pX Tax region. The signals of HTLV-1 proviral DNA pX Tax region were observed in lymphoma cells of T-cell and B-cell malignant lymphomas and Hodgkin's diseases and in histiocytes in low grade T-cell MLs (Hasui and Sato 1994). But there were no methods to evaluate adequately the specificity of the cISH employing the concatamer probe. On the other hand, the cISH employing the concatamer probe could not detect HTLV.1 proviral DNA, although the procedure included a step of denaturing DNA in sections.

Therefore, we tried to make several highly biotinylated probes corresponding to parts of HTLV-1 proviral DNA and to improve the procedures of DNA-RNA and DNA-DNA cISH for HTLV-1.

Materials and Methods

Material is the cell block paraffin sections of MT-2 cell line (given from Prof. Miyoshi I., Kochi Medical University, to Prof. Sonoda S, Kagoshima University). After one month culture with fetal calf serum, the MT-2 cells were packed in paper sac, fixed in buffered 10% formalin, processed in the tissue processor (Sakura, EPT120A) and embedded in paraffin. Three μm thick paraffin sections of the MT-2 cell block were adhered on the 3-aminopropyl-tretoxy silane-coated slide glasses.

Synthesis of highly biotinylated double-stranded DNA probes by polymerase chain reaction (PCR)

Biotinylated double-stranded DNA probes for ISH of HTLV-1 were produced by means of 2 times PCR (Fig. 1). The probes produced by this 2 times PCR were expected to be more specific for HTLV-1 proviral DNA than synthesized more or less 30bp-long oligo-nucleotide probes and to be more sensitive than probes end-labeled because bio-11-dUTP is the smallest molecule labeling DNA and is incorporated in multiple sites of thymidylate in DNA sequence of probes.

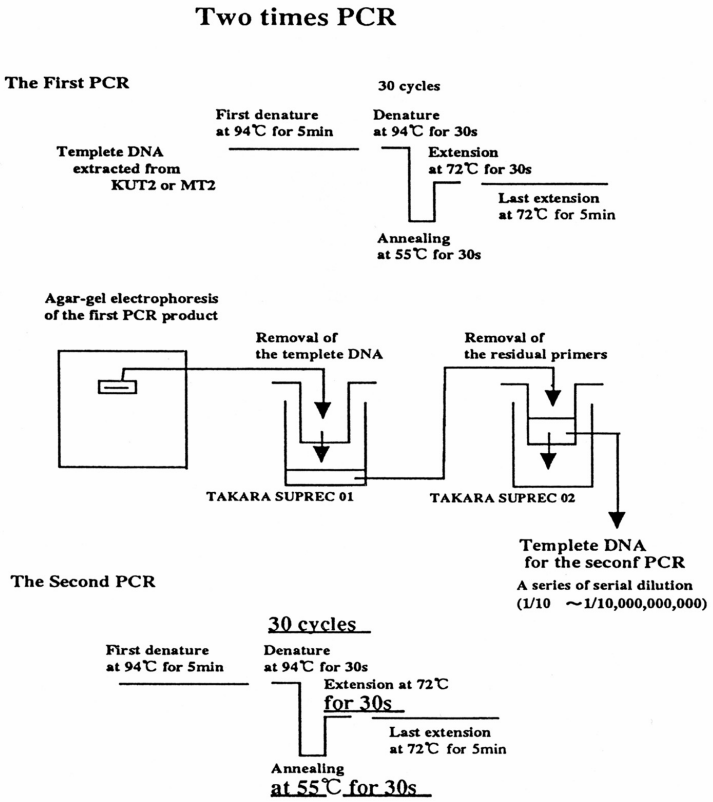


Fig. 1. Procedure of synthesizing highly biotinylated probes by means of 2 times polymerase chain reaction (PCR).

The first PCR was performed to get a pure template DNA for the Second PCR for producing biotinylated probe. The product of the first PCR was examined by agar-gel electrophoresis. The expected band on the gel was extracted by means of a microtube with filter (Suprec0l). Residual PCR primers were removed by processing the Solution with a microtubc with filter (Suprec02). The 10^{-1} to 10^{-10} diluted TE Solutionf of the extracted pure template DNA were once examined to produce enough product by the same protocol of the second PCR. Usually one (10^{-3} to 10^{-6}) of the template DNA Solution was the template DNA for the second PCR.

The first PCR produced template DNA comprising an expected region of HTLV-1 proviral DNA for the second PCR. Template DNA for the first PCR was extracted from MT-2 cell line of which DNA had at least 7 integrated copies of HTLV-I proviral DNA. Protocol of the first PCR comprised the first denature of DNA at 94℃ for 5 min, 30 cycles of denature at 94℃ for 30 sec, annealing at 55℃ for 30 sec and extension at 72℃ for 30 see and the followed extension at 72℃ for 5 min. A band of amplified DNA of agar-gel electrophoresis was extracted by using microtubes with filters (Suprec^{01} and Suprec^{02}, Takara Co.). Serial 10 times diluted extracted DNA-Tris ethylene-diamine tetraacetic acid EDTA solutions (TE) were examined to get a band at the expected length in agar-gel electrophoresis of product of the second PCR which was performed under the same protocol as the first PCR. The Selected one of the diluted amplified DNA TE Solutions was the DNA template for the PCR with bio-11-dUTP to synthesize highly biotinylated double-stranded DNA probe (Lo DYM et al. 1990).

Table 1 Biotinylated probes produced

Probe	Primes of PCR	Length
LTR	HTL119 - HTL120	717bp
Gag	HTL116 - HTL117	208bp
Pol 1	SK54 - SK55	118bp
Pol 2	HTL125 - HTL126	236bp
Env	HTL105 - HTL106	327bp
Tax	SK43 - SK44	159bp
Rex	HTL137 - HTL138	555bp
Control	Lambda bacteriophage	500bp

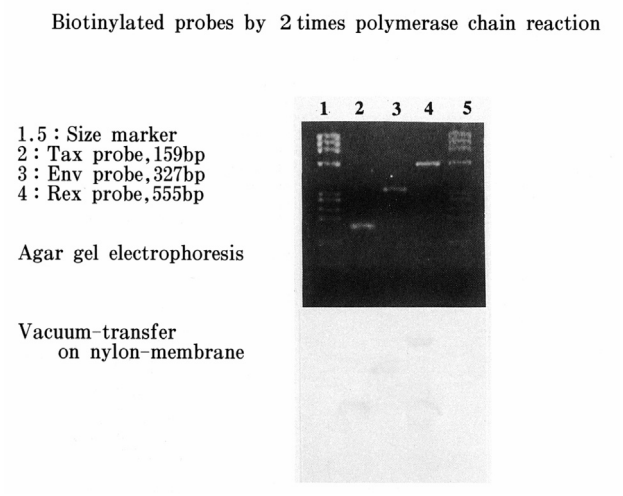


Fig.2. Agar-gel electrophoresiS of highly biotinylated probes (Tax, Env and Rex) and detection of the incorporation of bio-11-dUTP into the probes vacuum-transferred on nylon membrane.
The probes vacuum-transferred were visualized by means of streptavidine-biotinylated alkaline phosphatase system and new fucshin system. The reaction solutions of streptavidine and biotinylated alkaline phosphatase were I : I,000 0.05M Tris buffer pH 7.6. The reactions were performed in a hybridization bag. Each band of the probes were visualized on the nylon membrane.

Biotinylated double-stranded DNA probes synthesized were listed in Table 1. Figure 2 shows agar-gel electrophoresis of the biotinylated probes Tax, Env and Rex. Their bands vacuum-transferred on nylon membrane could be visualized by streptavidine-biotinylated alkaline phosphatase system (DAKO) and new fucshin-alkaline phosphatase reaction.

Procedures of cold in-situ-hybridization (cISH)

The original procedure of ISH for HTLV-1 was designed to detect both signals and proviral DNA sequence (Hasui and Sato 1994). Table 2 shows procedures of DNA-DNA and DNA-RNA cISH employed in this study.

Table 2 Procedure of cold in situ hybridization (cISH)

	Procedure for DNA-RNA ISH	Procedure for DNA-DNA ISH
Digestion	Proteinase K (10 μ g/ml) 37℃, 30 min.	Pepsin (200mg/ml, pH 2) 37℃, 30 min.
Denature		
for sections	-	incubate in a moist aluminum chamber at 200℃ for 10 min.
for probes	Incubate a probe microtube in boiling water for 5 min. followed by rapid cooling on crushed ice	-
ISH		
Solution	Hybridization buffer (Amersham RPN.131) with 50% formamide	Hybridization buffer (Amersham RPN.131) with 50% formamide addition of 5 mg/ml salmon sperm DNA
Probes	one of probes	7 probes cocktail
Temperature	45℃	45℃
Time	3 hours	3 hours
Post-ISH wash	2 times for 15 min. at 55℃ in 2xSSC and 0.01xSSC	2 times for 15 min. at 55℃ in 2xSSC and 0.01xSSC
Detection of probes		
Streptavidine-biotinylated ALP New Fucshin system	1 times at room temperature for 30 min.	1 times at room temperature for 30 min

Sections were dewaxed, digested by pepsin for DNA-DNA cISH or by proteinase K for DNA-RNA

cISH, post-fixed 4% paraformaldehyde phosphate buffer saline (PBS) and dried through 100% ethanol and in vacuum chamber.

Denature of DNA in sections and probes in DNA-DNA cISH was performed, by heating an aluminum box, in which the sections with hybridization solution were set above 150 m1 50% formamide 2x standard saline citrate (SSC), at 200℃ for 10 min, After the denature the aluminum box was incubated at 45℃ for 3 hours for hybridization. The hybridization solution comprised 50% formamide buffer (Amersham RPN.13l), a cocktail of the 7 biotinylated probes for HTLV-1 and 5mg/ml salmon sperm DNA.

In DNA-RNA cISH only probes in hybridization buffer (Amersham RPN. 131) containing 50% formamide were denatured, floating microtubes containing probes on boiling water for 5 min and cooling the microtubes on ice rapidly. Hybridization was performed, by incubating sections with hybridization covered by slide glass at 45℃ for 3 hours.

In both procedures for DNA-DNA and DNA-RNA cISH post-hybridization wash was carried out at 55℃. Hybridized probes were visualized by streptavidine-biotinylated alkaline phosphatase system and new fucshin System (DAKO).

Results

DNA-RNA cold in-situ hybridization (cISH) for HTLV-1

The DNA-RNA clSH of each probe could detect signals in cytoplasm of MT-2 cells (Fig. 3a to e). Signals of each sequence of LTR, Gag, Env and Tax HTLV-1 proviral DNA were recognized in nucleoli of a few MT-2 large cells (Fig. 3a, b, d and e). Only weak and faint stains were noted in cytoplasm and nucleolus in the DNA-RNA cISH of lambda bacteriophage 500bp probe (Fig. 3f). These weak and faint stains were the background stain of the DNA-RNA cISH in this study.

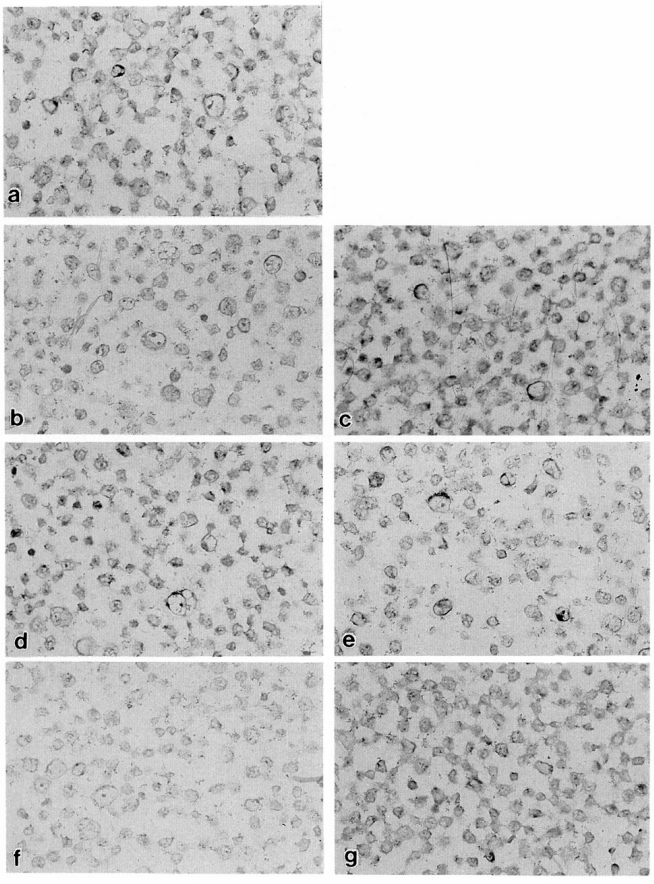


Fig. 3. DNA-RNA. cold in situ hybridization (cISH) for HTLV-1 in MT-2, employing biotinylated probes corresponding to each part of HTLV-1 provlral DNA.

a) LTR (717bp probe)
b) Gag (208bp probe)
c) Pol 1 (118bp probe)
d) Env (327bp probe)
e) Tax (159bp probe)
Each probe can detect signals corresponding to the Sequence of HTLV-I proviral DNA. The signals were recognized in cytoplasm of MT-2 cells. In a few MT-2 large cells nucleolar stain is also seen.

f) Lambda bacteriophage 500bp probe biotinylated.
In comparison with a) to e) of probes for HTLV-1, only weak stain is seen in cytoplasm and nucleoli.

g) cISH with hy.bridization buffer containing no probes.
Dense cytoplasmic stain is non-spccifie product of alkaline phosphatase reaction for 30 min at room temperature. (Original magnification on film : x 25)

A small number of T-cell and B-cell malignant lymphomas and Hodgkin's disease were examined by the DNA-RNA cISH of Env, Tax and Rex probes. In the DNA-RNA cISH of Tax and Rex probes, some lymphoma cells showed perinuclear cytoplasmic and dot-like stain in the T-cell pleomorphic lymphoma, adult T-cell leukemia/lymphoma (ATLL) type (Fig. 4b and c) and B-cell malignant lymphoma, centrocytic anaplastic type (Fig. 5b and c). In the DNA-RNA cISH of Env probe, some lymphoma cells showed cytoplasmic dot-like stain (Fig. 4a and Fig. 5a).

Fig.4. DNA-RNA cold in-situ hynbridization of Env, Tax and Rex probes in T-cell pleomorphic lymphoma, ATLL tyye.

a) Env
b) Tax
c) Rex

Signals of each part corresponding to the HTLV-I proviral DNA are seen in cytoplasm of large and medium-sized lymphoma cells. (Original magnification on fiim : x 50)

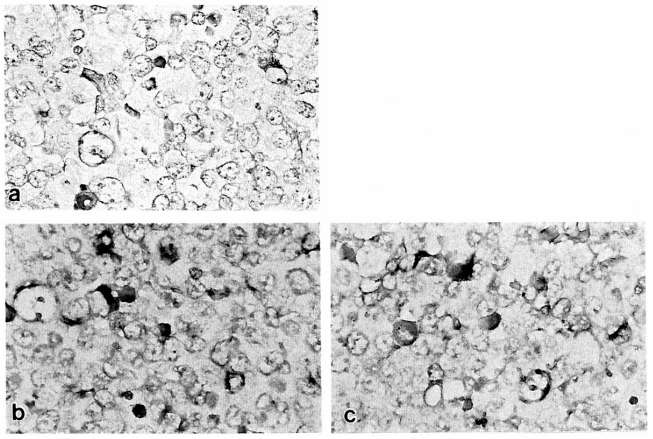
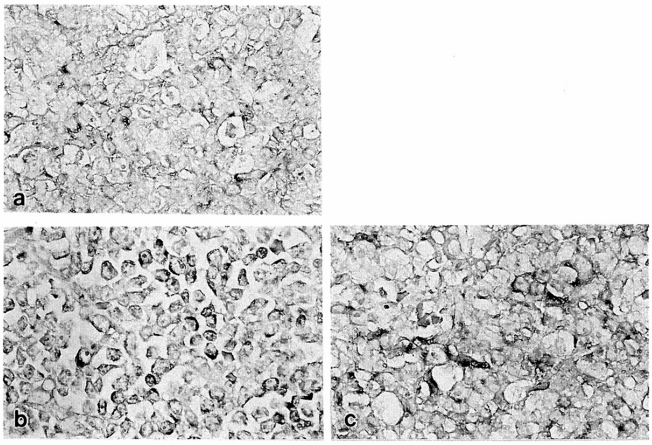


Fig. 5 DNA-RNA cold in situ hybridization of Env, Tax and Rex probes in B-cell malignant lymphoma, centrocytic anaplastic type, with a relation to HTLV-1 infection.

a) Env
b) Tax
c) Rex

Signals of each part corresponding to the HTLV-1 proviral DNA are seen in cytoplasm of lymphoma cells. (Original magnification on film: x50)



The DNA-RNA cISH of hybridization buffer containing no probes yielded cytoplasmic dense stain (Fig. 3g). Employing 17% polyvinyl alcohol as a solvent of the alkaline phosphatase reaction buffer, the background stain in cytoplasm of MT-2 cells was reduced in the DNA-RNA cISH of Tax probe and of the hybridization buffer containing no probes even when the alkaline phosphatase reaction was performed for 3 hours (Fig. 6).

DNA-DNA cold in-situ-hybridization (clSH) for HTLV-1

DNA-DNA cISH was performed according to the procedure as given in Table 2. A few MT-2 cells showed cytoplasmic stain but there was no obvious stain in any nuclei of MT-2 cells.

By elongation of the hybridization time to 3 days and of the alkaline phosphatase reaction time to overnight when 17% polyvinyl alcohol was used as the solvent of the reaction solution, the DNA-DNA cISH could show weak and fine dot-like stains on some nuclei of MT-2 cells (Fig.7).

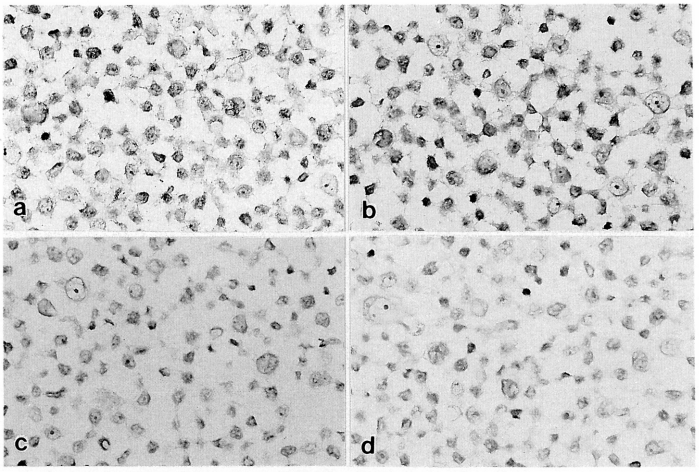


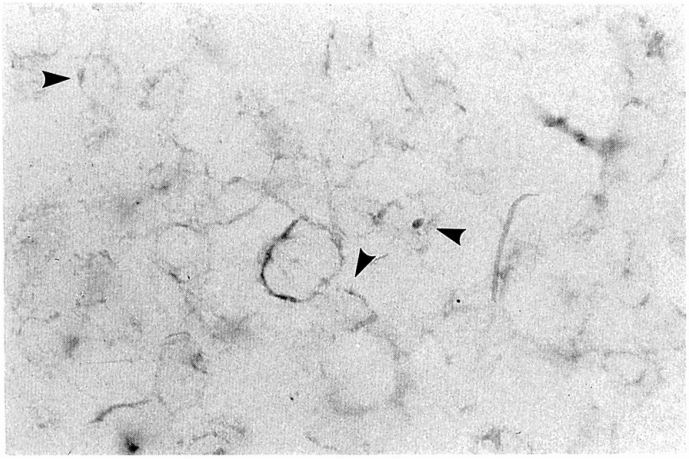
Fig.6. Non-specific prod.uct of alkaline phosphatase reaction for 3 hours at room temperature and effect of 17% polyvinyl alcohol solvent in the reaction in sections of DNA-RNA cold in situ hybridization. Alkaline phosphatase reaction without 17% polyvinyl alcohol in DNA-RNA cISH.with Tax probe (a) and without probes (b) : Cytoplasmic stains are seen in the both of DNA-RNA cISH wIth Tax probe (a) and without probes (b). Alkaline phosphataSe reaction with 17% polyvinyl alcohol in DNA-RNA cISH with Tax probe (c) and wIthout probes (d) : Only in DNA-RNA cISH with Tax probe (c) cytoplasmie stain is seen. Non-specific product of alkaline phosphatase reaction on cytoplasm is diminished under the effect of 17% polyvinyl alcohpl solvent. (0riginal magnification on film: x25)

Fig.7. DNA-DNA coIf in situ hybridization for HTLV-I in MT-2, elongating hybridization upto 3 days and employing 17% polyvlnyl alcohol alkalIne phosphatase reaction solution.

Weak and fine rot-like stains (arrows) were recognized in some nuclei of MT-2 cells.

Perinuclear stain in large cell located in the center is the signal rather than HTLV-1 DNA genome in cyt.plasm.

(Original magnification on film: x 100)



Discussion

Three kinds of mRNA, gag-pol mRNA, env mRNA and Tax/Rex mRNA, are produced in HTLV-1 infected cells, depending on the proviral DNA of HTLV-1 incorporated into DNA of the cells (Yoshida and Fujisawa 1992). The probes biotinylated and produced in this study corresponded to each sequence of HTLV-I proviral DNA, as shown in Fig. 8. The DNA-RNA cISH of these probes detected these 3 kinds of mRNAs in MT-2 cells, as shown in Fig. 3. As shown in Fig. 4 and 5, the DNA-RNA cISH of the probes for HTLV-1 proviral DNA env region and pX Tax/Rex region could detect signals in some of lymphoma cells of HTLV-1-related T-cell and B-cell malignant lymphomas as well as Hodgkin's disease (Hasui and Sato 1994). But the figures of lymphoma cells having the signals and of the signals were different each other. Because the probes for HTLV-1 proviral DNA pX Tax region and for pX Rex region must hybridize the same mRNA, the difference must be explained to be induced by the probe length. The signals of the Rex probe (555bp) were thought to be more specific for the mRNA than those of the Tax probe (159bp). On the other hand, the cISH of the Tax probe was thought to be more sensitive, because the mRNA exists also as its fragments in paraffin section.

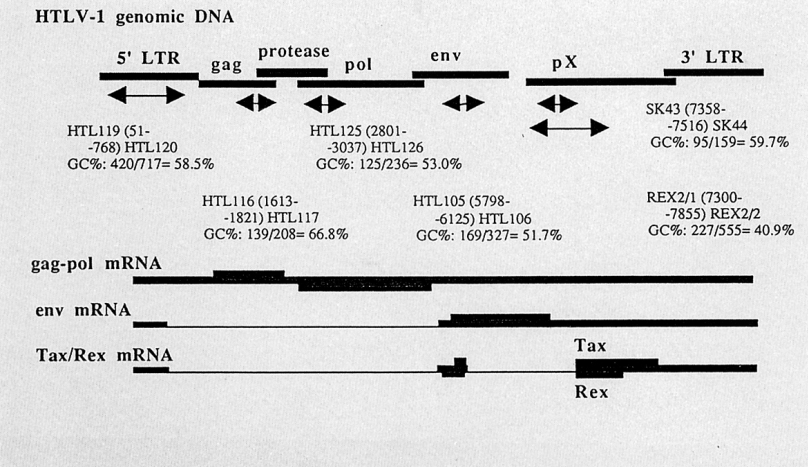


Fig. 8. Relationship among probes produced, proviral DNA and mRNAs of human T-Cell leukemia virus type I (HTLV-1)

These probes were double-stranded DNA and yielded sense and anti-sense single stranded DNA probes by means of denature. The DNA-RNA cISH of double-stranded DNA probes can not analyze the direction of DNA reading. Two times PCR may produce anti-sense or sense Single-stranded DNA probe, applying asymmetric PCR or one-side PCR to the second PCR in the two times PCR in Fig 1. If the single-stranded DNA probes could be applied to the DNA-RNA cISH, the direction of reading DNA will be analyzed. By analyzing the reading direction of the pX region, it will be indicated whether the retrograde reading of the pX region (Seiki et a1. 1983) may effect on the oncogenesis of HTLV-1-related malignant lymphomas.

Non-specific stain of alkaline phosphatase reaction was much weaker in DNA-RNA cISH with probes than in DNA-RNA cISH without probes. In order to block the non-specific alkaline phosphatase reaction, prehybridization-incubation with hybridization buffer containing salmon DNA was thought to be necessary in the DNA-RNA cISH, although the non-specific stain of alkaline phosphatase reaction could be diminished by employing 17% polyvinyl alcohol (Kiyama and Emson 1991) as reaction Solvent (Fig. 6). Because the DNA-RNA cISH of lambda bacteriophage 500bp probe showed weak and faint stain, the prehybridization-incubation was thought to be necessary to reduce the background stain in the DNA-RNA cISH.

On the other hand, the original procedure of cISH employed in the previous study (Hasui and Sato 1994) included a process of denature of DNA in sections by means of incubation in 70% formamide 2xSSC for 5 min at 70℃ followed by rapid cooling. In consequence of the previous study the original procedure detected signals of HTLV-1 proviral DNA pX region. The denature treatment was thought to effect on the sensitivity of the DNA-RNA cISH, although the high sensitivity of the original procedure was explained to be induced by the concatamer probe. The heat pretreatment was reported to make cISH more sensitive (Kiyama and Emson 1991).

The DNA-DNA cISH in this study can show only weak and faint dot-like stain of HTLV-I proviral DNA in some nuclei of MT-2 cells of which DNA has at least 7 copies of HTLV-I proviral DNA. But the DNA-DNA cISH in this study was not the expected method to be applied to see the integrated HTLV-1 proviral DNA in HTLV-1-infected cells in human tissue, because of the weak and faint dot-like stain. The dot-like stain itself was thought to detect the proviral DNA, because chromosome painting can show only fine dots on nuclei when the amount of DNA in one chromosome is extremely larger than the length of HTLV-l proviral DNA, although nuclear stain in DNA-DNA cISH employing probes labeled by random primers method was reported to detect HTLV-1 proviral DNA in the ATLL cells in the skin (Arai et a1. 1994). When it is true that one copy of HTLV-1 proviral DNA is incorporated in each nuclei of ATLL cells, the ratio of the nuclei with one dot stain in the DNA-DNA cISH must be proportional to the nuclear volume in the section (Hasui and Sato 1986).

We think that a success of DNA-DNA cISH for HTLV-1 depends on the development of the hybridized probe detection system of the extremely higher sensitiveness. One candidate of the methods may be the repeated reaction of streptavidine-biotinylated alkaline phosphatase system, because a couple of the reaction can be achieved in less than 20 min when semiautosystem of immunohistochemistry is applied to this procedure.

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