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Retroviral DNA Integration Directed by HIV Integration Protein in Vitro

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Efficient retroviral growth requires integration of a DNA copy of the viral RNA genome into a chromosome of the host. As a first step in analyzing the mechanism of integration of human immunodeficiency virus (HIV) DNA, a cell-free system was established that models the integration reaction. The in vitro system depends on the HIV integration (IN) protein, which was partially purified from insect cells engineered to express IN protein in large quantities. Integration was detected in a biological assay that scores the insertion of a linear DNA containing HIV terminal sequences into a λ DNA target. Some integration products generated in this assay contained five–base pair duplications of the target DNA at the recombination junctions, a characteristic of HIV integration in vivo; the remaining products contained aberrant junctional sequences that may have been produced in a variation of the normal reaction. These results indicate that HIV IN protein is the only viral protein required to insert model HIV DNA sequences into a target DNA in vitro.

RETROVIRUSES ENCODE A PROTEIN, IN protein, that is necessary for normal integration of retroviral DNA and efficient retroviral growth (*1, 2*). Studies of several retroviruses suggest that integration involves a coordinated set of DNA cleaving and joining reactions mediated by IN protein. First, the flush-ended linear DNA produced by reverse transcription is prepared for integration by IN protein–dependent cleavage, yielding recessed 3′ ends. In the Moloney murine leukemia virus (M-MuLV) system, production of these recessed ends in vivo depends on the viral IN function (*3, 4*); purified avian myeloblastosis virus (AMV) IN protein can carry out this reaction in vitro (*5*). Next, viral DNA is inserted into a DNA target, a reaction that also has been shown to depend on IN function in several retroviral systems in vivo (*2*). Analysis of a DNA intermediate of M-MuLV integration indicates that the recessed 3′ ends of the viral DNA are joined to the 5′ ends of a double-strand break made in the DNA target (*3, 6*); staggered cleavage of the target DNA is inferred from the short duplication of target DNA that flanks the integrated proviral DNA. Integration is completed by removal of unpaired bases at the 5′ ends of the viral DNA and repair of the resulting single-stranded gaps, presumably by cellular DNA repair enzymes, yielding the short duplication of the target DNA at the point of insertion. Each retroviral integration system generates a target duplication of characteristic length, ranging from 4 to 6 bp (*1*).

The human immunodeficiency virus (HIV) genome encodes an IN protein that is similar in sequence to those of other retroviruses (*7*), and the HIV integration function is known to be required in vivo for

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Table 1. Integration of mini-HIV in the presence of partially purified HIV IN protein. Line 1 presents the results of 21 independent reactions; line 2 presents the results of 6 independent reactions. Integration reactions contained 10 μl of partially purified HIV IN protein or the same volume of the corresponding protein preparation made from uninfected cells, 1 μg of mini-HIV DNA, 1.5 μg of λ DNA, bovine serum albumin (0.1 mg/ml) RNase A (0.1 mg/ml), RNase T1 (2 U/ml), *Xenopus* histone H1 (20 μg/ml), protein HU (2 μg/ml) (*22*), 130 mM potassium glutamate, 20 mM Hepes (pH 7.5), 5 mM $MgAc_2$, 10 mM dithiothreitol, 0.05% NP-40, 10% dimethyl sulfoxide, and 10% glycerol in a final volume of 50 μl. Reaction mixtures were incubated at 0°C for 1 hour, then 8 μl of 30% polyethylene glycol was added, and the reactions were incubated for 30 min at 30°C.

Integration protein	Colonies	Phage
HIV IN	71	5.9×10^9
Control insect cell extract	0	1.3×10^9

viral replication (*8*). The enzymology of IN protein has been difficult to examine directly in any retroviral system, because the cell-free integration systems established to date utilize complex nucleoprotein particles derived from the viral core as a source of integration activity. These particles are known to contain several viral proteins and may contain host proteins as well (*9–11*).

As a first step in establishing a fully defined system that carries out integration of HIV DNA in vitro, we have overexpressed and partially purified active HIV IN protein. We transferred the DNA sequence encoding this protein to the chromosome of a baculovirus (AcMNPV) and used this recombinant virus (561-3) to express IN protein in insect cells (Sf9) (*12–14*). We prepared extracts of Sf9 cells from 561-3–infected and control cultures and compared them by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Only extracts from 561-3–infected cells contained a prominent protein with a molecular weight of 32,000, the expected size of HIV IN protein. This protein partitioned in the insoluble fraction from lysates of Sf9 cells infected with 561-3. Washing of this material with high-salt buffer yielded a preparation enriched in the species with a molecular weight of 32,000 (Fig. 1A) (*14*), which we subsequently confirmed to be HIV IN protein by amino acid sequencing (*15*).

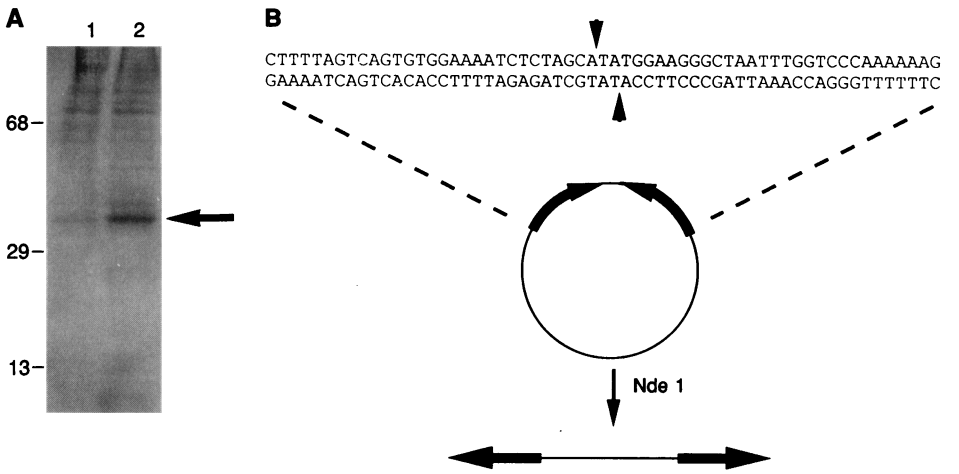

Fig. 1. Preparation of HIV IN protein and its substrate mini-HIV. (**A**) SDS-PAGE analysis of extracts of insect cells (Sf9) expressing HIV IN protein. (Lane 1) Extract of insoluble proteins from uninfected Sf9 cells. (Lane 2) Extract of insoluble proteins from Sf9 cells infected with 561-3, the baculovirus that contains the HIV IN protein coding region. The arrow marks the position of HIV IN protein. Dashes with numbers indicate the mobilities of marker proteins with the indicated sizes ($\times 10^{-3}$). (**B**) Structure of the mini-HIV DNA. The sequence of the oligonucleotide duplex matching the HIV LTR ends (the 5′ end of the left LTR and the 3′ end of the right LTR of pNL4-3) (*23*) is shown at the top. Digestion of a plasmid containing this duplex with Nde I yields the linear mini-HIV DNA. Mini-HIV DNA contains the predicted ends of the linear HIV DNA precursor for integration except for the 5′-TA-3′ extension at the 5′ ends, which is inferred to be 5′-AC-3′ in the precursor in vivo (*24*).

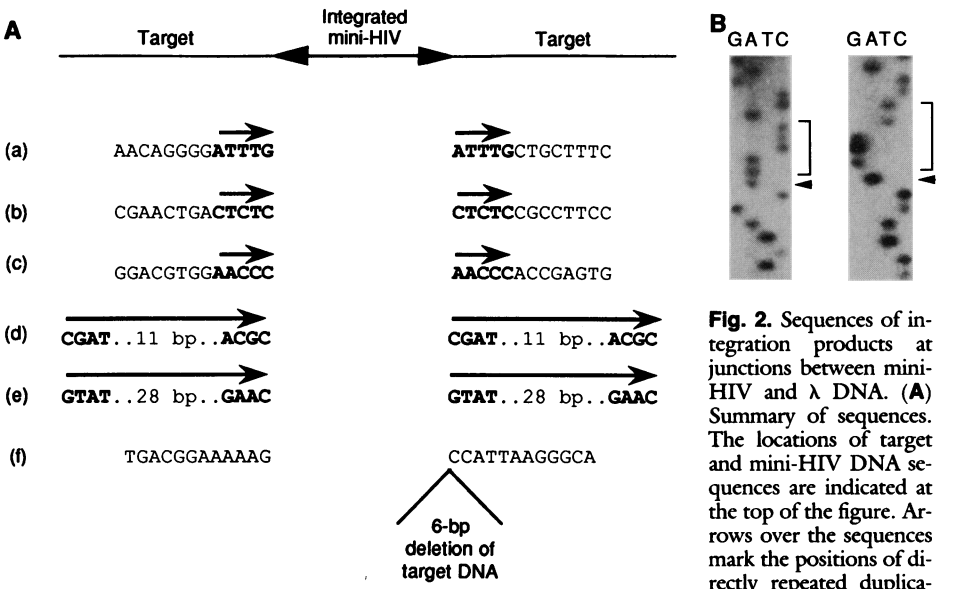

Fig. 2. Sequences of integration products at junctions between mini-HIV and λ DNA. (**A**) Summary of sequences. The locations of target and mini-HIV DNA sequences are indicated at the top of the figure. Arrows over the sequences mark the positions of directly repeated duplications in the target DNA, and duplicated sequences are shown in bold letters. Each mini-HIV contains the expected 5′-TG and CA-3′ sequences at each border with target DNA (not shown). Integration events (a to c) contain 5-bp target duplications characteristic of HIV integration in vivo. Events (d) to (f) have unusual junction sequences: (d) contains a 19-bp duplication, (e) contains a 36-bp duplication, and (f) contains a 6-bp deletion of the λ DNA target adjacent to the point of insertion. Integration products were sequenced by the Sanger method (*25*) with the primers described in (*10*). (**B**) Sample sequence determination. The autoradiographs display the sequence from each end of integration event (c) in (A). The arrow marks the last base of mini-HIV. The bracket marks the duplication of host DNA. Note that the primers used to sequence each end anneal to opposite DNA strands, so the sequence duplication at one end of mini-HIV reads as an inverted copy of the complementary sequence at the other end.

We monitor integration by means of a biological assay that scores the insertion of a "mini-HIV" model substrate into λ DNA. Mini-HIV consists of a linear DNA molecule bearing ends that resemble the predicted ends of the unintegrated HIV DNA (Fig. 1B)(*16*). The 3′ ends of mini-HIV are recessed, thereby bypassing the need for cleavage by the predicted long terminal repeat (LTR)–specific nuclease activity of IN protein. We score insertion of mini-HIV into λ DNA by packaging the reaction products into phage heads in vitro and infecting an *Escherichia coli* strain containing a λ prophage with the resulting phage particles. Bacteriophage λ cannot grow on the lysogenic strain, but λ DNA containing integrated mini-HIV can persist as a plasmid because mini-HIV contains the pBR322 origin of replication. Since mini-HIV also contains genes conferring resistance to ampicillin and tetracycline, we can detect integration events by plating infected cells on selective plates and scoring the number of colonies that arise.

We add several small basic proteins to the in vitro integration reaction. From a biological assay similar to that used for these studies, it was found that extracts of uninfected NIH 3T3 cells stimulated M-MuLV DNA integration in vitro (*10*). It was subsequently found that ribonuclease (RNase) A or RNase T1 stimulated M-MuLV DNA integration almost as well, and that protein HU provided a slight further stimulation. Histone H1 provided a small additional stimulation in some experiments. These proteins were therefore included in our assays of HIV integration and were found in control experiments to stimulate product recovery at least tenfold. The effects of these proteins have not been assessed separately in the HIV system. The significance of the stimulatory effects of these proteins in the biological assay system is unclear: M-MuLV

integration can now be monitored by a simple physical assay in vitro, and addition of these small basic proteins does not detectably stimulate integration (*17*).

Assays of reactions containing partially purified HIV IN protein, mini-HIV DNA, and λ DNA in a suitable reaction mixture yielded recombinant-containing bacterial colonies (Table 1). However, when extracts of insoluble proteins from insect cells (Table 1) or insect cells infected with wild-type AcMNPV (*18*) were substituted for the extract containing HIV IN protein, these control assays yielded no recombinants.

We determined the DNA sequences of the junctions between mini-HIV and λ DNA at each end of six independent recombinants. Three of these recombinants contained 5-bp duplications of the target DNA at the point of insertion (Fig. 2A, a to c), and the other three recombinants contained a 19-bp duplication, a 36-bp duplication, and a 6-bp deletion of the target DNA (Fig. 2A, d to f).

The following observations indicate that the DNA insertion reactions detected in our biological assay have the attributes of the reaction directed by HIV IN protein. First, control extracts of Sf9 cells do not support detectable integration. Second, in all cases, both ends of the mini-HIV DNA are joined to the target DNA after the CA-3′ dinucleotide that marks the ends of the integrated HIV proviral DNA (*19*). Third, the sites of insertion in the target DNA show no obvious sequence specificity, as is characteristic of integration of HIV and other retroviruses in vivo. Fourth, in three of the six sequenced recombinants, the integrated mini-HIV is flanked by a 5-bp duplication of target DNA, a characteristic of correct HIV integration in vivo (*19*). The conclusion that our in vitro system depends on HIV IN protein is strengthened by results of a parallel study of M-MuLV integration in vitro. We find that integration of a mini-M-MuLV DNA similarly depends on addition of M-MuLV IN protein partially purified from Sf9 cells, and recombinants contain 4-bp duplications of target DNA, characteristic of M-MuLV integration in vivo (*20*) and not 5-bp duplications (*21*).

Although the isolation of aberrant integration products indicates that our reactions in vitro sometimes deviate from the normal reaction pathway, we note that the aberrant recombinants could result from a simple variation of the normal integration scheme. In the normal reaction, coordinated attack by the two HIV DNA ends joins viral DNA to breaks on each strand of the target DNA that are separated by 5 bp (Fig. 3A, top and middle). Repair of this intermediate, presumably by cellular enzymes, generates the 5-bp duplications of target DNA found to flank HIV proviruses (Fig. 3A, bottom). The products containing longer duplications or a deletion of target DNA can be understood as resolution products of intermediates resulting from uncoupled attack by each viral DNA end at unrelated sites on each strand of the target DNA, as shown in Fig. 3B.

It has been suggested that a viral core-like structure containing the DNA substrate and multiple viral proteins might be necessary for integration [reviewed, for example, in (*1*)]. Our results indicate that, on the contrary, HIV IN protein can insert HIV DNA sequences into a DNA target in vitro in the absence of other viral proteins. Note, however, that the ends of mini-HIV are recessed, thereby bypassing the need for the specific cleavage of the LTR termini by the IN protein, and so our data do not address the question of whether viral proteins in addition to the IN protein are required for the cleavage step. Although the efficiency of the in vitro integration system used here is low and the framework of the high molecular weight complex may improve the efficiency and fidelity of integration, our results suggest that HIV IN protein contains all the enzymatic machinery required for the integration reaction.

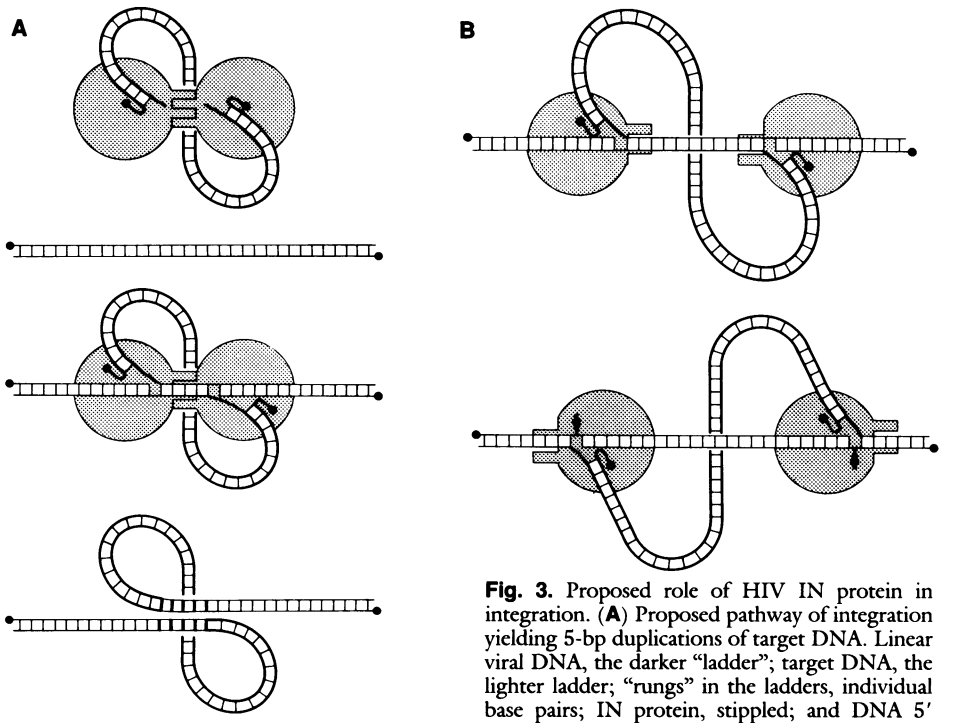

Fig. 3. Proposed role of HIV IN protein in integration. (**A**) Proposed pathway of integration yielding 5-bp duplications of target DNA. Linear viral DNA, the darker "ladder"; target DNA, the lighter ladder; "rungs" in the ladders, individual base pairs; IN protein, stippled; and DNA 5′ ends, dark dots. The integration-competent complex composed of IN protein and linear viral DNA is shown (A, top). Integration proceeds by joining of the recessed 3′ ends of viral DNA to protruding 5′ ends of a staggered break in a DNA target (A, middle). This mechanism has been confirmed for the related M-MuLV system (*3, 6*) and our observation that a linear mini-HIV DNA can integrate in vitro to give the normal integration product is consistent with the view that the same mechanism holds for HIV. The integration intermediate is then resolved by melting of the target DNA between the joints with viral DNA, nucleolytic removal of unpaired viral DNA 5′ ends, and repair of the resulting gaps (A, bottom), thus producing 5-bp duplications of the target DNA (indicated by thick rungs). The latter reactions are probably carried out by cellular DNA repair enzymes during normal integration and occur in our biological assay either in the λ DNA packaging extract or in bacteria after infection. (**B**) Uncoupled attack by each end of mini-HIV on target DNA could yield abnormal intermediates leading to longer target duplications or deletions. The intermediate pictured at the top of (B) would result from uncoupled attack joining mini-HIV DNA ends to longer-than-usual protruding 5′ ends of a staggered break in the target DNA. Melting of target DNA between the junctions with viral DNA followed by DNA repair would yield a target duplication of greater than normal length. The intermediate shown in (B), at the bottom, would result from uncoupled attack linking viral 3′ ends to recessed 5′ ends of a staggered break in the target DNA. Endonucleolytic cleavage at the positions indicated by the arrows (or melting of this DNA segment accompanied by degradation of the resulting unpaired ends) would delete a segment of the target DNA. Deletions and duplications might also be formed if the two ends of mini-HIV interact with sequences on two different λ DNA molecules. The idea that uncoupled attack occurs in the HIV system suggests that an IN protein moiety can bind to each viral DNA end; thus the IN protein is pictured as a multimer in the normal integration scheme in (A).

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13. The recombinant baculovirus expressing IN protein was made as follows: the 0.7-kb Pvu II to Bsp MI fragment of pNY5′ (*23*) containing the central portion of the DNA that encodes IN protein was isolated by standard methods, and duplex oligonucleotide linkers were ligated to both ends. One synthetic duplex DNA restored the 5′ part of the IN protein coding region and added (i) the AUG necessary for translation, since the viral IN protein is derived by proteolytic cleavage of a polyprotein precursor; (ii) a 9-bp region matching the 9 bp just 5′ of the initiation codon of the baculovirus *polyhedrin* gene; and (iii) a Bam HI cohesive end. To facilitate future manipulations of the DNA, base 4313 was changed from A to G to generate a Bsp MI site. A second synthetic duplex DNA restored the 3′ sequence of the gene and added a Bam HI cohesive end. This Bam HI fragment was then ligated into the Bam HI site of pAc373 (*12*) to make pMK561. The recombinant baculovirus expressing IN protein was generated by recombination in vivo between pMK561 and *Autographa californica* nuclear polyhedrosis virus DNA after cotransfection as described (*12*). After plaque purification, one isolate, 561-3, was used for expression of IN protein.
14. The HIV IN protein was prepared as follows: Sf9 cells were infected with 561-3 as described (*12*), and HIV IN protein was isolated from infected cells after 3 days of incubation in 150-cm^2 flasks. All the purification steps were carried out at 4°C. Cells adhered to the flask after removal of the medium, so that cells could be washed with 5 ml of 150 mM potassium glutamate and 20 mM tris (pH 8), and then lysed by addition of 5 ml of buffer A containing 100 mM potassium glutamate [buffer A is 30 mM tris (pH 8), 10 mM $MgAc_2$, 1% NP-40, and 10% glycerol]. Lysates were centrifuged 10 min, at 13,000*g*. The pellet was washed in 2.5 ml of buffer A containing 400 mM potassium glutamate by homogenization with a Kimax manual homogenizer. This material was centrifuged again for 10 min at 13,000*g*, and the insoluble pellet was resuspended in 1 ml per flask of starting cells of buffer A containing 100 mM potassium glutamate. Control extracts of uninfected or AcMNPV-infected Sf9 cells were prepared in exactly the same way.
15. The HIV IN protein, purified by SDS-PAGE, was sequenced from the amino terminus in an Applied Biosystems Model 471A Protein Sequencer as described by P. Matsudaira [*J. Biol. Chem.* **262**, 10035 (1987)]. The first amino acid was found to be methionine, and the next 19 amino acids matched the sequence of the amino terminus of HIV IN protein isolated from infected cells, as determined by M. M. Lightfoote *et al.* [*J. Virol.* **60**, 771 (1986)] (data not shown).
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Inhibition of T Cell Receptor Expression and Function in Immature CD4$^+$CD8$^+$ Cells by CD4

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Most immature CD4$^+$CD8$^+$ thymocytes express only a small number of T cell receptor (TCR) molecules on their surface, and the TCR molecules they do express are only marginally capable of transducing intracellular signals. TCR expression and function was not intrinsically low in immature CD4$^+$CD8$^+$ thymocytes, but was found to be actively inhibited by CD4-mediated signals. Indeed, release of CD4$^+$CD8$^+$ thymocytes from CD4-mediated signals resulted in significant increases in both TCR expression and signaling function. These results suggest that, in CD4$^+$CD8$^+$ cells developing in the thymus, increased TCR expression and function requires release from CD4-mediated inhibition.

THYMIC SELECTION OF THE DEVELoping T cell repertoire occurs in immature CD4$^+$CD8$^+$ thymocytes, with the fate of individual thymocytes determined by the T cell antigen receptors (TCR) they express (*1*). Thus, it seems paradoxical that most CD4$^+$CD8$^+$ thymocytes express few TCR molecules that are only marginally capable of transducing intracellular signals (*2*). Identifying the regulatory mechanisms involved in either inducing or inhibiting TCR expression and function in developing CD4$^+$CD8$^+$ thymocytes would enhance our understanding of T cell differentiation. The TCR^{lo} phenotype of CD4$^+$CD8$^+$ thymocytes is due to the low fractional survival of newly synthesized and assembled TCR complexes in these cells, which can be quantitatively increased by in vivo administration of monoclonal antibody (MAb) to CD4 (*3*). CD4, when cross-linked, activates tyrosine kinases that phosphorylate various intracellular substrates, including TCR-ζ (*4, 5*), a subunit of the TCR complex that appears to be important for both TCR expression and TCR signal transduction (*6*). We now directly assess the influence of CD4-mediated signals on TCR expression and function in immature CD4$^+$CD8$^+$ thymocytes. TCR expression and function in immature CD4$^+$CD8$^+$ thymocytes was inhibited by CD4-mediated signals, and the presence of these inhibitory signals correlated with the reported phosphorylation status of TCR-ζ in these cells (*5*).

Experimentally, TCR expression can be polyclonally increased in CD4$^+$CD8$^+$ thymocytes either by in vivo administration of MAb to CD4 (*7*) or by in vitro culture of thymocytes in single-cell suspension (*8*). In vitro, TCR^{lo} thymocytes spontaneously increase their expression of TCR when cultured in single-cell suspension at 37°C but not at 4°C (Fig. 1A), with TCR profiles of thymocytes from suspension cultures at

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