PERGAMON

Leukemia Research 27 (2003) 575–582

Leukemia Research

www.elsevier.com/locate/leukres

Measurement of *SIL–TAL1* fusion gene transcripts associated with human T-cell lymphocytic leukemia by real-time reverse transcriptase-PCR

John D. Curry [a], Martyn T. Smith [b,*]

[a] *Division of Immunology, Department of Molecular and Cellular Biology, University of California at Berkley, 439 Life Sciences Addition, Berkeley, CA 94720-3200, USA*

[b] *Division of Environmental Health Sciences, School of Public Health, University of California, 216 Earl Warren Hall, Berkeley, CA 94720-7360, USA*

Received 11 February 2002; accepted 4 October 2002

Abstract

TAL1 disruption at 1p32 [del(1p)] is a common rearrangement in the development of T-cell acute lymphocytic leukemia (T-ALL). The del(1p) are usually interstitial 90 kb deletions placing *TAL1* under control of the SCL interrupting locus (*SIL*) gene forming the *SIL–TAL1* fusion product. A reverse transcriptase real-time PCR assay to quantify *SIL–TAL1* fusion genes is described. A *SIL–TAL1* fusion gene RNA transcript was built that permitted absolute standard curves to be generated. Sensitivity of the RT-PCR assay was determined to be 10 cells (CEM cell line) in 10^6 human lymphocytes. Peripheral blood lymphocytes from 10 healthy adults and 10 neonates were assayed. None of the samples showed any *SIL–TAL1* expression. However, when lymphocytes from three adults were cultured in vitro the *SIL–TAL1* transcript was detectable in the RNA isolates. No *RAG2* expression was detected in these expanded samples, suggesting that the clones bearing the *SIL–TAL1* fusion gene may have existed at low levels prior to the ex vivo expansion.

Keywords: *SIL–TAL1*; Real-time PCR; Lymphocytic leukemia; Childhood; Translocation

1. Introduction

TAL1 is a positive regulator of erythroid differentiation [1] and normal expression of the *TAL1* gene occurs in hematopoietic, endothelial and brain tissues [2]. Within the hematopoietic system a lineage restricted expression of *TAL1* occurs in erythroid, mast, megakaryocytes and progenitor cells [3], but not in T cells [4]. Disruption of the *TAL1* gene on chromosome 1p32 [del(1p)] is a common rearrangement in the development of T-cell acute lymphocytic leukemia (T-ALL) [5]. Approximately 25% of all childhood T-ALLs bear specific *TAL1* gene rearrangements [6]. These 90 kb interstitial deletions place the coding part of the *TAL1* gene under the control of the first non-coding exon of the SCL interrupting locus (*SIL*) gene. Aberrant V(D)J recombinase activity is thought to be responsible [7] resulting in a gain of function mutation in T-ALL. These tumor-specific rearrangements cannot be detected cytogenetically [8]. To solve this problem, a conventional reverse transcriptase (RT)-PCR method for the detection of all *TAL1* and novel *TAL1* deletion variants has been described [9]. This RNA-based method, however, is not quantitative. A quantitative method for measuring *SIL–TAL1* fusion gene transcripts would be desirable for the detection of minimal residual disease in T-ALL. Such an assay could also be used for the specific detection of aberrant V(D)J recombinase activity at these genes. Such aberrant activity has been related to the induction of lymphomas and lymphocytic leukemias and is currently assessed by a semi-quantitative DNA-based assay for inversion-(7) [10,11].

Translocation t(14;18), between the *BCL-2* proto-oncogene and JH immunoglobulin gene region, has been detected at low frequencies in the blood of healthy individuals [12–14]. *MLL–AF4* fusion gene transcript [t(4;11)] expression has been reported in presumably healthy individuals [15], although there exists some controversy over this report [16,17]. Very low frequencies of the Philadelphia chromosome, t(9;22), involving the breakpoint cluster region (*bcr*) and the *abl* gene, have also been reported in healthy individuals [18]. The frequency of these t(9;22) transcripts demonstrated a direct correlation with subject age. Here, we report

* Corresponding author. Tel.: +1-510-642-8770; fax: +1-510-642-0427.
E-mail address: martynts@uclink.berkeley.edu (M.T. Smith).

0145-2126/03/$ – see front matter
PII: S0145-2126(02)00260-6

on part of our on-going efforts to determine if other types of translocations are also present in peripheral blood lymphocytes.

A recently described DNA-based assay for the detection of *SIL–TAL1* deletions (type I and II splice variants) is reportedly capable of detecting 1 leukemic cell in 100,000 normal cells using a first round PCR followed by real-time PCR for quantification [19]. However, as a fusion gene is likely to express more than one copy of the fusion mRNA, an assay detecting mRNA is likely to be more sensitive than one utilizing genomic DNA. In addition, *SIL–TAL1* detection by RT-PCR is preferable as all deletion variants can be detected with a single set of primers [20].

In this report, we describe the development of a sensitive RNA-based real-time RT-PCR system for the detection of *SIL–TAL1* fusion transcripts. This assay was then used to determine the background frequency, if any, of the *SIL–TAL1* fusion event in human peripheral lymphocyte populations. As the interstitial deletions which generate *SIL–TAL1* are thought to be mediated by the illegitimate activity of V(D)J recombinase, cord blood lymphocyte populations from neonates were also examined. Three of the adult samples were cultured in vitro and aliquots of the expanding T-cell populations were assayed. After 7 days of expansion, *SIL–TAL1* fusion gene transcripts were detected in RNA extracted from these expanded samples. *RAG2* expression was also assayed in these expanded samples, but none was found, which potentially indicates that the detected *SIL–TAL1* fusion events pre-existed in the original lymphocyte populations. To the best of our knowledge, this is the first indication that this fusion event might exist in healthy individuals. The *SIL–TAL1* transcript copy number was also determined for the commonly used standard, the CEM cell line.

2. Methods

2.1. Construction of SIL–TAL1 plasmid and RNA runoff transcripts

RNA was extracted from the CCRF-CEM (CCL-119, ATCC) cell line (1×10^7 cells) using a RNA minicolumn (Qiagen). First strand cDNA was produced using methods described [20] with the following modifications. RNA (5 μg) was primed with 100 ng of TAL1-E3 primer (Table 1) (MWG Biotech), 0.5 mM dNTPs (Roche), five units of Stratascript® reverse transcriptase (Stratagene), and one unit of RNase inhibitor (Eppendorf) for 50 min at 42 °C followed by 5 min at 95 °C. From the initial cDNA strand, a smaller double stranded DNA product was generated using PCR primers SIL-A and TAL1-B. PCR conditions were as described [20] and produced several different sized products, one of them being a 371 bp fragment. This type II splice variant PCR product was cloned into a pCR®2.1–TOPO® plasmid (Invitrogen).

To determine which plasmids contained a correctly orientated and sized insert, colonies were screened as follows. Using the M13 forward (−20) primer which is just 3′ to the T7 promoter sequence of the plasmid, and a *TAL1*-specific reverse primer (TAL-D), 1 μl of the overnight culture was directly assayed with a 20 μl PCR (dNTPs, 30 ng of forward and reverse primer, and two units of AmpliTaq™ polymerase (Applied Biosystems) with the supplied buffer (A). Reactions were cycled 35 times (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min). Cultures that generated a correctly sized product (266 + 112 bp of flanking plasmid sequence) were selected. Isolated plasmid DNA was sequenced by automated fluorescent sequencing and the sequences compared with the published *SIL–TAL1* fusion product sequence (SIL exon Ia (M74558), *TAL1* exons III–VI (S53245)). A single error-free plasmid was subsequently used to generate runoff RNA transcripts.

Large amounts of runoff RNA transcripts were produced using the MegaShortScript™ T7 kit following the manufacturer's directions (Ambion). To eliminate contaminating plasmid DNA, the runoffs were processed through a Qiagen RNA minicolumn and the product was then restricted with *Hae*III (several sites exist between the real-time primers), then treated with DNase I, and passed through a second RNA minicolumn. Real-time PCR (below) was used to directly screen the runoff RNA transcripts to ensure that no residual plasmid DNA remained. Quantity of the runoff transcript was initially determined by spectrophotometry and then confirmed using a RiboGreen™ RNA quantification kit (Molecular Probes).

Table 1
Sequences of the primers used for reverse transcription and real-time PCR reaction

Primer code	5′-Position (size)	Sequence (5′–3′)	Reference
TAL1-B	384 (18)	CGCGCCCAGTTCGATGAC	[20]
TAL1-E3	451 (19)	CGTCGCGGCCCTTTAAGTC	[20]
TAL1-D	280 (18)	CCGCGTCCCGTCCCTCTA	[20]
TAL1-D-R	281 (17) Reverse	CGCGTCCCGTCCCTCTA	This study
SIL-A	82 (21) Forward	TCCCGCTCCTACCCTGCAA	[20]
SILTAL1-D	231 (21) Detection	GCGGCCGCTCGGTCATCCT	This study
hRAG2-F	(20)	ATACCTGGTTTAGCGGCAAA	[27]
hRAG2-R	(22)	GCCAGCCTTTTTGTCCAAAGAA	[27]

5′-Positions are as noted by van Dongen et al. [20].

2.2. Design of real-time primers

Real-time PCR primers were manually designed and then tested using Primer Express Software. Guidelines for primer design were as suggested by Applied Biosystems. PCR primers originally designed by van Dongen et al. [20] met these requirements and were used, except that one 5′ cytosine residue was removed from primer TAL1-D and renamed TAL1-D-R (Table 1). Thus, PCR primers SIL-A and TAL1-D-R are able to detect the three different splice variants as originally described [20]. PCR primers were proofed using the selected *SIL–TAL1* fusion gene plasmid. A TaqMan™ probe (SILTAL1-D) was designed such that it anneals to the 5′-end of *TAL1* exon IV. The probe was labeled with the VIC™ and 6-carboxytetramethylrhodamine (TAMRA) dyes (Applied Biosystems). Probe labeled with 6-carboxyfluorescein, succinimidyl ester (FAM) was obtained later as this dye was considerably less expensive (MWG Biotech). Primer and probe concentrations were optimized following the manufactures directions (Applied Biosystems).

2.3. Reverse transcription and real-time PCR

First strand cDNA, generated from runoff RNA transcripts, was used to construct a standard curve for *SIL–TAL1* fusion gene expression. To more closely mimic a real-life reaction, human total RNA (Stratagene) was also added to the reactions (0.5 μg/20 μl reaction). RT reaction cocktails (1× buffer, 0.5 mM dNTPs, one unit of RNase inhibitor, five units of Stratascript® RT, 5 ng TAL1-B and 5 ng B2MG RT primer (described below) in a 20 μl volume) were incubated at 25 °C for 5 min, 42 °C for 50 min, and finally 95 °C for 5 min. Random hexamers can also be used instead of these two specific primers, but the TAL1-B primer did provide smaller Ct values, indicating better RT efficiencies (data not shown).

Real-time PCR reactions using 5 μl of the completed RT reaction were performed as directed by the manufacturer with the following modifications. Reaction volumes were decreased from 50 to 20 μl to economize reagent use. PCR primers TAL1-D-R and SIL-A were added at 100 ng each, while the SILTAL1-D TaqMan™ primer was just 10 ng (again this economized the expensive probe). Reactions were then subjected to 5 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 95 °C for 15 s and 60 °C for 60 s. Data was collected using an ABI Prism 7700 real-time PCR machine and analyzed with Applied Biosystems sequence detection software (Version 1.7). After analysis, real-time PCR data can be expressed as cycle thresholds (Ct) which are determined by the software. Where Ct data is not presented, the software analysis package has been utilized to generate the values shown.

2.4. Standard curves

Standard curves were prepared using serial dilutions of the *SIL–TAL1* fusion gene RNA runoff transcripts, ranging from 50 ng to 50 fg per RT reactions, with 0.5 μg of background RNA. RT reactions were prepared in replicates for each standard curve point. To determine the limit of the assay's sensitivity two additional standard curves were prepared. For the first, whole RNA was obtained from a series of limiting dilutions placing 10^{-1} to 10^{4} CEM cells into 10^{6} freshly obtained human lymphocytes. This situation more closely resembles the real-life situation of a leukemic clone contained within a normal population of cells. A second standard curve mixed cell equivalence amounts of RNA (4.0 pg of whole RNA per resting T-cell) at 5000, 500, 50, 5 and 0.5 CEM cells in a background of a million cells (RNA equivalents, 4 μg of human colon RNA). These RNA mixtures were then assayed by RT-PCR.

Beta-2-microglobulin (*β2MG*) mRNA expression was used as a standardization gene, and permitted RNA quantification and efficiency of the RT reaction to be determined. Primers for *β2MG* PCR were as previously described [21]. A *β2MG* RT primer was added to each RT cocktail mixture, and subsequently, a 2 μl portion of the cDNA cocktail was added to a real-time PCR assay. Briefly that assay contained; 10 μl universal master mix (2×) (Applied Biosystems), 60 ng each of the forward and reverse primers, and 6 ng of a 2′,7′-dichloro-6-carboxy-4,7-dichlorofluorescein (TET) and TAMRA-labeled probe (Operon). These β2MG Ct values determined the actual amount of RNA assayed. Reported *SIL–TAL1* values are corrected for that amount of RNA.

2.5. V(D)J recombinase RAG2 expression assay

A cDNA mixture was prepared as detailed above, but 120 ng of random hexamer (Gibco-BRL) was used to prime the mRNA molecules. A single round of conventional PCR was used to detect *RAG2* cDNA molecules. The 20 μl reaction contained 2 μl of the cDNA reaction, 100 ng of forward and reverse *RAG2*-specific PCR primers (Table 1), 0.8 mM dNTPs, one unit of AmpliTaq™ Gold with 1× supplied buffer. An initial 95 °C for 5 min melting step was followed by 35 rounds of 60, 72 and 95 °C, each for 30 s. After completion, 5 μl of the reaction was loaded into a 0.75% ethidium bromide stained agarose gel. A 200 bp product indicated that there was *RAG2* expression.

2.6. Cells and cell culture

Human cell lines were obtained from ATCC and grown as directed. Total cellular RNA was prepared using Qiagen RNA minicolumns and assayed for the expression of the *SIL–TAL1* fusion genes, the *β2MG* gene and the *RAG2* gene. The Reh cell line was used as a positive control for the expression of the *RAG2* gene and was not tested for *SIL–TAL1* expression. RNA samples from 10 healthy adults and 10

neonates were obtained from the Ficoll separated mononuclear cell (MNC) fraction of whole blood collected under an IRB approved human subjects protocol. Additionally, three of the aforementioned adult MNC samples (approximately 20×10^6 cells) were stimulated with phytohemagglutinin (PHA; 0.25 μg/ml, Wellcome Diagnostics) and interleukin-2 (IL-2) (five units/ml, Gibco-BRL) in RPMI 1640 with 10% fetal bovine serum. The expanding T cells were cultured for 9 days, and samples for RNA preparation withdrawn at days 3, 5, 7 and 9. RNA samples were demonstrated to be free of potential DNA contamination by directly performing real-time PCR on them. These samples were then assayed for *SIL–TAL1* fusion gene expression as described, but in a larger RT reaction volume (100 μl) containing 5 μg of RNA.

3. Results

3.1. Generation of a standard curve

Real-time RT-PCR assays are a rapid and accurate method for the quantification of fusion gene transcripts. Accurate standard curves are essential for the precise quantification of gene expression when employing any real-time PCR method. Extremely significant *SIL–TAL1* fusion gene standard curves ($R^2 = 0.99$, d.f. $= 25$, $P < 0.000001$) were generated using the constructed runoff RNA transcripts (Fig. 1). The reproducibility of the assay described was demonstrated as there was little or no difference between standard curves prepared in different experiments (likelihood ratio test: $X^2 = 0.31$, d.f. $= 2$, $P = 0.81$).

3.2. Sensitivity of the assay

Detection sensitivity of the RT-PCR assay was determined utilizing two distinct methods. The first method placed several known amounts of isolated CEM cell line RNA (containing the *SIL–TAL1* fusion gene transcript) into the RNA equivalent of one million cells. Each point consisted of three independent RT-PCR reactions. By way of comparison, an identical experiment was performed, but without adding the background RNA to the RT reaction. Effects of the background RNA can be seen in Fig. 2, where the Ct values for each of the four points were significantly (as noted by non overlapping standard errors) decreased as compared to the identical reactions containing the 10^6 cells RNA background. In both cases the last point, containing five CEM cell RNA equivalents was detectable, however, only one of the three identical reactions produced a detectable signal. This indicates the sensitivity of the assay as determined with this methodology.

The other method that was employed to determine the assay's sensitivity was to mix resting human lymphocytes with known numbers of CEM cells. RNA was then extracted and assayed. This method closely resembles the first method, but now also accounts for the RNA extraction process. RNA yields from resting lymphocytes were noted to be very low, and great numbers of lymphocytes had to be used to obtain suitable amounts of RNA for each standard curve point. For

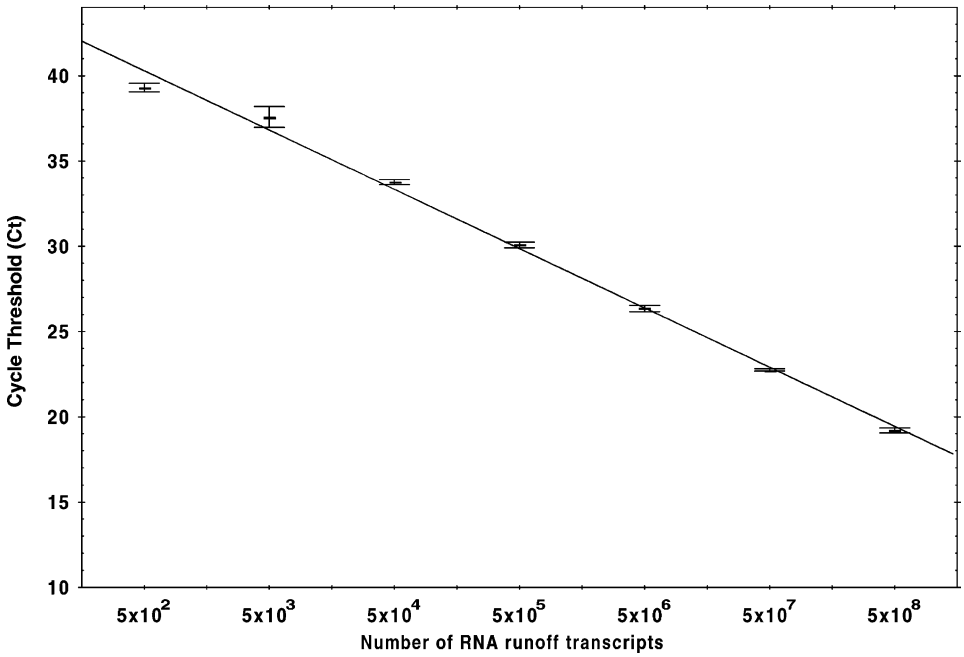

Fig. 1. Linear regression analysis yielding the standard curve for the *SIL–TAL1* fusion gene runoff transcripts. Mean Ct values are indicated by bars with standard errors (whiskers). Each point is the mean of results taken from four identical RT-PCR reactions. The line demonstrates a high level of significance with an R^2 value of 0.99, d.f. $= 25$ and $P < 0.000001$.

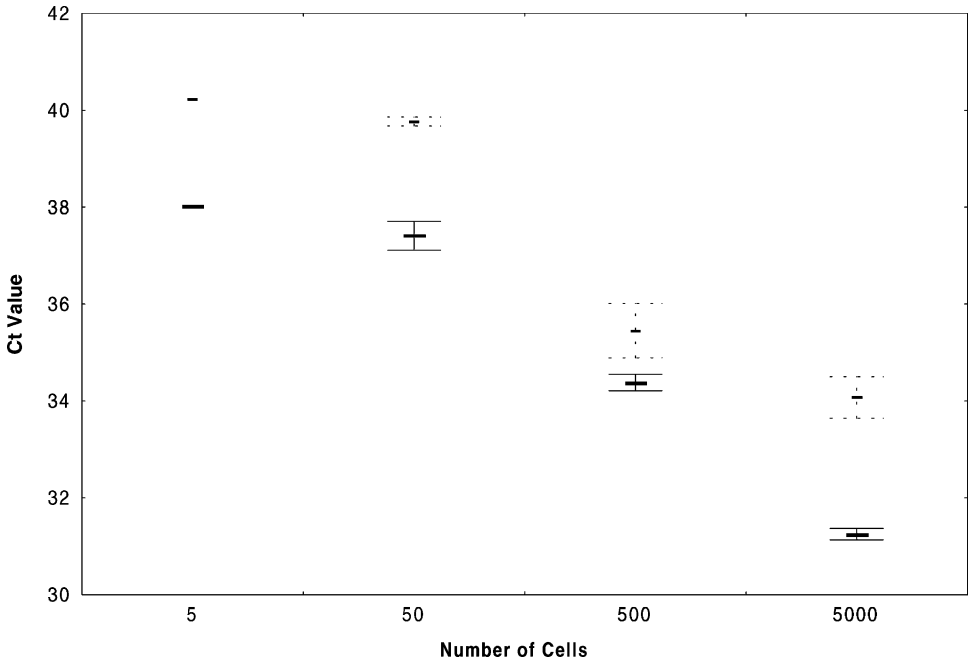

Fig. 2. Detection limits of the RT-PCR assay for the *SIL–TAL1* fusion gene. CEM RNA was either mixed into background RNA (dashed, and short bars) equal to 10^6 cells or not (solid, and wider bars). Mean Ct values are indicated by bars with standard errors (whiskers). Each point is the mean of results obtained from three identical RT-PCR reactions. The RNA equivalent of five CEM cells, bearing a total of 40 *SIL–TAL1* fusion genes could be detected. A zero cell control is not shown as no Ct values could be obtained from such a control.

this reason, this methodology was not used further to test the resolution. Fig. 3 shows the real-time data generated by this method, where 1 CEM cell in a background population of 10^5 resting lymphocytes was detectable.

3.3. SIL–TAL1 expression in the CEM human cell line

Minor differences in the RNA amounts assayed, due either to measurement (by spectrophotometer) or pipette error, were accounted for by standardization against the *β2MG* gene. Identical amounts of human colon RNA were repeatedly assayed in each experiment and the results consistently yielded the same *β2MG* Ct values (16.94 ± 0.07).

The molecular weight of the *SIL–TAL1* fusion gene RNA transcript including the flanking plasmid sequences (insertion site to *Sac*I restriction site and to the T7 promoter site) is 161,944.4 g/mol. Thus, 1 ag contains 3.847 transcripts (10^{-18} g/161,944.4 × 6.23 × 10^{23}). As 500 ng of total CEM RNA, which originated from 125,000 (4 pg RNA per cell), was determined to contain 2.9×10^5 ag of the fusion transcript, then each CEM cell can be calculated to contain approximately 9 transcripts (Table 2). The human lung cancer cell line (CI-H460) was found not to contain any expression level of *SIL–TAL1* fusion gene.

3.4. SIL–TAL1 expression in human lymphocytes from the general population

No expression of the *SIL–TAL1* fusion gene was detected in any of the 10 adult lymphocyte populations resting unstimulated in the G_0 state. Of the 10 cord blood samples assayed, none were found to express any detectable amount of the fusion gene.

3.5. Effect of lymphocyte expansion on SIL–TAL1 transcript detection

Lymphocytes from three of the previously screened adults were grown in culture under PHA and IL-2 stimulation, promoting the rapid expansion of the T cells. A 10-fold increase in the population sizes was noted after 9 days of culture (data not shown). Samples of the cultures taken at days 0, 5, 7 and 9, were subjected to real-time RT-PCR analysis of *SIL–TAL1* expression. None of the 0- or 5-day cultures displayed any expression of fusion gene. However, after 7 and 9 days, the *SIL–TAL1* fusion gene transcripts were detected in the cultures of all three subjects. The RT-PCR experiments were repeated and the mean number of transcripts detected per μg of input RNA is shown (Table 3).

For every experiment multiple negative controls were utilized, and never indicated any contaminating signals. Experimental design was such that potential contaminating templates were physically separated from the areas where the RT-PCR set up was performed. PCR products were never a contamination issue for two reasons: first the real-time PCR assay utilized dUTP during PCR product synthesis, and the TaqMan™ Master mix contained a uracil–DNA–*N*-glycosylase which would initially destroy any templates containing UTP. Secondly, and more convincingly, was the fact that the PCR product containing

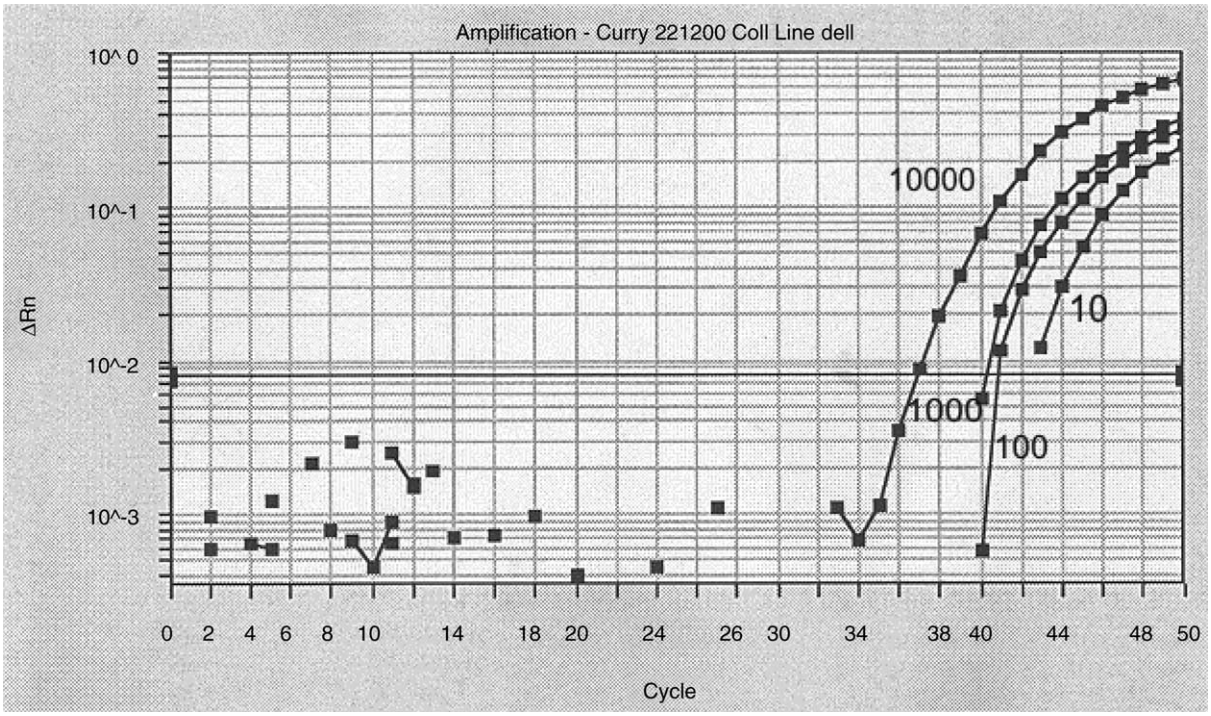

Fig. 3. Detection limits of the RT-PCR assay using RNA extracted from CEM cells diluted into resting human lymphocytes. Freshly obtained human lymphocytes (10^6) were mixed with 10^{-1} to 10^4 CEM cells, RNA extracted, and assayed for the *SIL–TAL1* fusion gene. The limits of the sensitivity were 10 CEM cells in 10^6 lymphocytes. The *y*-axis is a measure of the reporter dyes' fluorescence. The horizontal line indicates where the cycle thresholds will be read from. Single dots below the horizontal line represent noise.

reaction tubes were never opened and were safely discarded after the real-time PCR analysis.

3.6. V(D)J recombinase RAG2 expression

RAG2 expression was assayed for all the human cell lines and reported as either present or absent (Table 2). The Reh cell line (CRL-8286) was used as a positive control for the *RAG2* expression assay, while both the colon RNA and lung cancer fibroblast cell line served as negative controls. *RAG2* expression was always found in the Reh and CEM cell lines but never in the two negative controls. Assaying RNA from the adult time course experiments for *RAG2* expression was negative for each time point.

Table 2
SIL–TAL1 fusion and *RAG2* gene expression

Cell line ATCC #	Corrected *SIL–TAL1* fusion transcripts (ag) per 500 ng of whole RNA	*SIL–TAL1* transcripts per cell	*RAG2* detected
CEM CCL-119	$2.934 \pm 0.684 \times 10^5$	9.0	Yes
NCI-H460, HTB-177	None detected	0.0	No
Reh CRL-8286	–	–	Yes
Colon RNA	None detected	0.0	No

Table 3
Expression of the *SIL–TAL1* fusion gene transcript in expanding (PHA and IL-2 stimulated) human T-cell cultures. Data from three healthy adults (aged 25, 26 and 35 years) determined during two separate RT-PCR experiments

Day	*SIL–TAL1* (mean ± S.E.) transcripts per μg of whole RNA
0	None detected
5	None detected
7	7900 ± 1700
9	6400 ± 2600

4. Discussion

Sensitivity of the single round real-time PCR assay described here is considerably improved compared to the previously reported assay employing a nested RT-PCR design [14]. Detection sensitivity, as determined from the standard curve data, was 5 ag (19 copies) of transcript within a background of 500 ng total cell RNA. When mixing CEM total cell RNA into million cell RNA equivalents, the assay was able to detect five cells within one million. However, the sensitivity decreases two fold when actual CEM cells were mixed with normal human lymphocytes and the extracted RNA assayed.

Plasmid DNA containing the fusion gene sequence has previously been used to generate real-time standard curves.

While the use of plasmid DNA to generate curves has relevance when solely quantifying target DNA sequences, it does not equate well when attempting RNA quantification. A standard curve for RNA quantification must account for the low efficiency of the RT reaction [22], where the RNA is first transcribed into cDNA. Thus, use of fusion gene runoff RNA transcripts to generate a reliable real-time standard curve realizes a true measure of the RT-PCR assay. Standard curves constructed with runoff transcripts as demonstrated here have exceptional reproducibility and extremely little variation. Most of the variability is likely to arise from inaccuracies in volume pipetting, in particular the loading of a fraction of the RT volume into the real-time PCR volume. Considerable reductions in these inaccuracies can be realized through the use of highly accurate and reproducible electronic pipettes [23]. In addition, the real-time PCR reaction volumes can be loaded directly onto the entire RT reaction volume (20 μl). This of course requires much larger PCR volumes, but yields highly reproducible results.

Resting adult lymphocyte populations yielded no expression of the *SIL–TAL1* fusion gene. Lymphocyte populations from neonates (cord blood) were also assayed, as V(D)J is quite active in the lymphocytes at this stage of life, and thus could potentially act aberrantly. Such is the case with the hypoxanthine–guanine phosphoribosyltransferase (*hprt*) gene, a surrogate gene used for mutation detection, at which V(D)J has been demonstrated to aberrantly act [24]. Indeed, V(D)J mediated *hprt* deletions are more commonly observed in neonates than in adults [25,26]. However, from the 10 neonates lymphocyte populations analyzed, none demonstrated any *SIL–TAL1* expression. It is likely that the current sensitivity of the assay was insufficient to detect such rare events in either of these two populations. In addition, the population sizes examined here are rather small, but were meant only as a cursory examination. Further examination of larger populations, perhaps with greater sensitivity, is warranted.

Finding expression of the *SIL–TAL1* fusion gene in the expanded adult lymphocyte cultures was striking. From the data at hand, we are left to wonder the origin of the fusion transcripts, which clearly must be generated from the chromosome 1 interstitial deletion. Our reason for expanding the cultures in the first instance was to obtain more RNA. RNA extraction from resting lymphocytes generally yields rather low quantities (1.0 pg per cell ($0.37 \pm$ S.D.)), but stimulated and growing cells, produced more RNA (2.8 pg per cell ($2.7 \pm$ S.D.)) (means test: $P = 0.04$, one-sided). Also, our thinking was that the lymphocyte expansion might clone out any cells bearing the interstitial deletion which has been shown to provide a growth advantage.

After 5 days of culture and a greater than five-fold increase in population size, no fusion transcripts were detected. However, after 7 and 9 days of culture, a considerable number of transcripts were detected. Rapid expansion of the cell cultures might have been a factor in inducing the deletion event. However, as this event is V(D)J recombinase mediated, and the resting T-cell population will have completed that phase of there life cycles, this alternative is unlikely. No *RAG2* expression could be detected in any of these expanded lymphocytes populations, which discounts the possibility that the deletion event occurred during expansion of the lymphocyte populations. With this in mind, we suggest that the deletion event existed a priori and that the T-cell culture conditions favored cloning of a sub-population bearing this interstitial deletion. Again, the possibility of contamination is discounted as none of the negative controls, which were carried out in parallel with these experiments yielded any indications of contamination. Similar cultures of several neonate cord blood samples for 3–4 days (as part of another experiment) revealed one culture that contained 2100 *SIL–TAL1* transcripts per μg RNA. We are unable at this time to account for this unusual observation, although in this neonate case illicit V(D)J recombinase activity is more likely to be a factor than in the adults. We were unfortunately unable to attempt a sequence characterization of the actual deletion junctions. Such analysis would have definitively identified the origins of the fusion genes detected in the expanded adult samples.

Complexities of these seemingly simple expanded primary T-cell populations are likely to mask the origins of the *SIL–TAL1* fusion genes that may have been detected. The *SIL–TAL1* fusion gene is likely to be exceedingly rare in healthy individuals, but the possibility of its existence cannot be ruled out. While the transcript copy number for the CEM cell line was obtainable, the determination of the transcript copy number in a single resting T-cell bearing an interstitial deletion remains elusive. Indeed, the biological activity of this fusion gene in pre-leukemic cells in vivo remains unknown. A larger population study with a more sensitive assay would likely be more definitive in defining the background frequency of this event in human populations.

Acknowledgements

We gratefully acknowledge Drs. Nina T. Holland and Rustam Turakulov for their considerable efforts in organizing and obtaining the neonate and adult samples. Professor Mark Schlissel is thanked for his critical comments and V(D)J recombinase expertise. We thank Yen Wu for the RNA extractions. Dr. Cliona McHale's encouragement, advice and criticism were warmly received. Mathew C. Glaser is acknowledged for his editorial help in preparation of this manuscript. The laboratory work associated with this paper was performed by Dr. Curry as part of his post-doctoral research fellowship. Dr. Smith conceived and funded the study. Both authors contributed to the production of this manuscript. This research was funded by NIH grants P42ES04705 and P30ES01896 from the National Institute of Environmental Health Sciences. JC is trainee of the Health Effects Component of the University of California Toxic Substances Program. J.D. Curry developed the

concept, design, collected and assembled the data, analyzed the data, drafted the manuscript and gave final approval. M.T. Smith contributed to the concept, design, provided critical revision of the manuscript, provided the necessary funding and gave final approval.

References

[1] Aplan PD, Nakahara K, Orkin SH, Kirsch IR. The SCL gene product: a positive regulator of erythroid differentiation. EMBO J 1992;11:4073–81.

[2] Visvader J, Begley CG, Adams JM. Differential expression of the *LYL*, *SCL* and *E2A* helix–loop–helix genes within the hemopoietic system. Oncogene 1991;6:187–94.

[3] Cross MA, Heyworth CM, Murrell AM, Bockamp EO, Dexter TM, Green AR. Expression of lineage restricted transcription factors precedes lineage-specific differentiation in a multipotent hemopoietic progenitor cell line. Oncogene 1994;9:3013–6.

[4] Chen Q, Cheng JT, Tasi LH, Schneider N, Buchanan G, Carroll A, et al. The *tal* gene undergoes chromosome translocation in T-cell leukemia and potentially encodes a helix–loop–helix protein. EMBO J 1990;9:415–24.

[5] Aplan PD, Lombardi DP, Ginsberg AM, Cossman J, Bertness VL, Kirsch IR. Disruption of the human SCL locus by "illegitimate" V(D)J recombinase activity. Science 1990;250:1426–9.

[6] Brown L, Cheng JT, Chen Q, Siciliano MJ, Crist W, Buchanan G, et al. Site-specific recombination of the *tal-1* gene is a common occurrence in human T-cell leukemia. EMBO J 1990;9:3343–51.

[7] Janssen JW, Ludwig WD, Sterry W, Bartram CR. *SIL–TAL1* deletion in T-cell acute lymphoblastic leukemia. Leukemia 1993;7:1204–10.

[8] Bash RO, Hall S, Timmons CF, Crist WM, Amylon M, Smith RG, et al. Does activation of the *TAL1* gene occur in a majority of patients with T-cell acute lymphoblastic leukemia? A Pediatric Oncology Group study. Blood 1995;86:666–76.

[9] Delabesse E, Bernard M, Landman-Parker J, Davi F, Leboeuf D, Varet B, et al. Simultaneous *SIL–TAL1* RT-PCR detection of all *tal*(d) deletions and identification of novel *tal*(d) variants. Br J Haematol 1997;99:901–7.

[10] Lipkowitz S, Garry VF, Kirsch IR. Interlocus V–J recombination measures genomic instability in agriculture workers at risk for lymphoid malignancies. Proc Natl Acad Sci USA 1992;89:5301–5.

[11] Garry VF, Tarone RE, Kirsch IR, Abdallah JM, Lombardi DP, Long LK, et al. Biomarker correlations of urinary 2,4-D levels in foresters: genomic instability and endocrine disruption. Environ Health Perspect 2001;109:495–500.

[12] Liu Y, Hernandez AM, Shibata D, Cortopassi GA. *BCL-2* translocation frequency rises with age in humans. Proc Natl Acad Sci USA 1994;91:8910–4.

[13] Limpens J, Stad R, Vos C, de Vlaam C, de Jong D, van Ommen GJ, et al. Lymphoma-associated translocation t(14;18) in blood B cells of normal individuals. Blood 1995;85:2528–36.

[14] Yasukawa M, Bando S, Dolken G, Sada E, Yakushijin Y, Fujita S, et al. Low frequency of *BCL-2*/J(H) translocation in peripheral blood lymphocytes of healthy Japanese individuals. Blood 2001;98:486–8.

[15] Uckun FM, Herman-Hatten K, Crotty ML, Sensel MG, Sather HN, Tuel-Ahlgren L, et al. Clinical significance of *MLL–AF4* fusion transcript expression in the absence of a cytogenetically detectable t(4;11)(q21;q23) chromosomal translocation. Blood 1998;92:810–21.

[16] Trka J, Zuna J, Hrusak O, Michalova K, Muzikova K, Kalinova M, et al. No evidence for *MLL–AF4* expression in normal cord blood samples. Blood 1999;93:1106–1107 [discussion, p. 1108–1110].

[17] Kim-Rouille MH, MacGregor A, Wiedemann LM, Greaves MF, Navarrete C. *MLL–AF4* gene fusions in normal newborns. Blood 1999;93:1107–8.

[18] Biernaux C, Loos M, Sels A, Huez G, Stryckmans P. Detection of major *bcr–abl* gene expression at a very low level in blood cells of some healthy individuals. Blood 1995;86:3118–22.

[19] Chen X, Pan Q, Stow P, Behm FG, Goorha R, Pui CH, et al. Quantification of minimal residual disease in T-lineage acute lymphoblastic leukemia with the *TAL1* deletion using a standardized real-time PCR assay. Leukemia 2001;15:166–70.

[20] van Dongen JJ, Macintyre EA, Gabert JA, Delabesse E, Rossi V, Saglio G, et al. Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. Leukemia 1999;13:1901–28.

[21] Pallisgaard N, Clausen N, Schroder H, Hokland P. Rapid and sensitive minimal residual disease detection in acute leukemia by quantitative real-time RT-PCR exemplified by t(12;21) *TEL–AML1* fusion transcript. Genes Chromosomes Cancer 1999;26:355–65.

[22] Curry JD, McHale C, Smith MT. Low efficiency of the Moloney murine leukemia virus reverse transcriptase during reverse transcription of rare t(8;21) fusion gene transcripts. Biotechniques 2002;32(4):768–75.

[23] Curry JD, McHale C, Smith MT. Factors influencing real-time RT-PCR results: application of real-time RT-PCR for the detection of leukemia translocations. Mol Biol Today 2002;3:79–84.

[24] Fuscoe JC, Zimmerman LJ, Lippert MJ, Nicklas JA, O'Neill JP, Albertini RJ. V(D)J recombinase-like activity mediates *hprt* gene deletion in human fetal T lymphocytes. Cancer Res 1991;51: 6001–5.

[25] Fuscoe JC, Zimmerman LJ, Harrington-Brock K, Burnette L, Moore MM, Nicklas JA, et al. V(D)J recombinase-mediated deletion of the *hprt* gene in T lymphocytes from adult humans. Mutat Res 1992;283:13–20.

[26] Finette BA, Poseno T, Albertini RJ. V(D)J recombinase-mediated *HPRT* mutations in peripheral blood lymphocytes of normal children. Cancer Res 1996;56:1405–12.

[27] Wang QF, Lauring J, Schlissel MS. The c-*myb* binds to a sequence in the proximal region of the *RAG2* promoter and is essential for promoter activity in T-lineage cells. Mol Cell Biol 2000;20:9203–11.