Genes encoded within 8q24 on the amplicon of a large extrachromosomal element are selectively repressed during the terminal differentiation of HL-60 cells

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Abstract

Human acute myeloblastic leukemia HL-60 cells become resistant to differentiation during long-term cultivation. After 150 passages, double minute chromosomes (dmins) found in early-passaged cells are replaced by large extrachromosomal elements (LEEs). In a DNA library derived from a purified fraction of LEEs, 12.6% (23/183) of clones were assigned to 8q24 and 9.2% (17/183) were assigned to 14q11 in the human genome. Fluorescence *in situ* hybridization (FISH) revealed a small aberrant chromosome, which had not been found in early-passaged cells, in addition to the purified LEEs. We determined that each LEE consisted of six discontinuous segments in a region that extended for 4.4 Mb over the 8q24 locus. Five genes, namely, *Myc* (a proto-oncogene), *NSMCE2* (for a SUMO ligase), *CCDC26* (for a retinoic acid-dependent modulator of myeloid differentiation), *TRIB1* (for a regulator of MAPK kinase) and *LOC389637* (for a protein of unknown function), were encoded by the amplicon. Breaks in the chromosomal DNA within the amplicon were found in the *NSMCE2* and *CCDC26* genes. The discontinuous structure of the amplicon unit of the LEEs was identical with that of dmins in HL-60 early-passaged cells. The

difference between them seemed, predominantly, to be the number (10 to 15 copies per LEE *versus* 2 or 3 copies per dmin) of constituent units. Expression of the *Myc*, *NSMCE2*, *CCDC26* and *LOC389637* and *TRIB1*genes was constitutive in all lines of HL-60 cells and that of the first four genes was repressed during the terminal differentiation of early-passaged HL-60 cells. We also detected abnormal transcripts of *CCDC26*. Our results suggest that these genes were selected during the development of amplicons. They might be amplified and, sometimes, truncated to contribute to the maintenance of HL-60 cells in an undifferentiated state.

1. Introduction

Gene amplification, a cytogenetic abnormality identified in many cancer cells, can result in the elevated expression of certain oncogenes via an increase in the copy number of such genes or, occasionally, in the generation of abnormal fused genes that can promote malignancy, especially in late-stage cancer (for reviews, see refs. in [1] and [2]). The amplified gene(s) is often located on a piece of extrachromosomal DNA, such as a double minute chromosome (dmin), which consists of repeats of a certain chromosomal region (an amplicon). Many different models have been proposed to explain the origin of amplicons. In some models, abnormal replication that has escaped from cell-cycle control produces extra copies of the DNA of amplified genes; in other models, undesirable recombination events during the repair of damaged DNA initiate abnormal amplification [3, 4].

When human acute myoblastic leukemia HL-60 cells [5] are cultured, early-passaged cells contain dmins on which the *Myc* gene is amplified [6, 7, 8]. In a previous report, we described

some significant features of the chromosomal amplification of *Myc*, as follows [9]. Structures larger than dmins, which had been observed in early-passaged HL-60 (designated HL-60DM) cells, emerged after approximately 30 passages and gradually replaced the dmins in cells during subsequent continuous culture. These large extrachromosomal elements (LEEs) were eventually detectable in all cells after 150 passages, when only a few dmins remained. The number of LEEs varied from one to four per cell but was never greater than four. The length of each LEE was approximately 25% of that of human chromosome 21 [10]. The proto-oncogene *Myc* was amplified on both dmins and LEEs and there was no detectable integration of *Myc* into other chromosomes, even after more than 200 passages. The LEE was an unusual structure that lacked the alphoid sequence that is required for the generation of a constitutive centromere [10] and, as a consequence, each LEE could easily be distinguished from homogeneously staining regions that were integrated into other chromosomes [10, 11].

Decreases of numbers of dmins in HL-60DM cells, which are early-passaged cells (prior to

passage 28), result in growth arrest and apoptosis, and 3-5% of HL-60DM cells differentiate spontaneously in culture [12, 13]. In cultures of HL-60 cells, the number of spontaneously differentiated cells decreases in parallel with increases in numbers of LEE-positive cells and no spontaneously differentiated cells can be detected in cultures of LEE-positive and dmin-negative, long-passaged (designed HL-60LEE) cells [13]. Terminal differentiation of HL-60LEE cells cannot be induced by dimethylsulfoxide (DMSO), but the DMSO-induced growth arrest of these cells is reversed upon withdrawal of DMSO. By contrast, DMSO induces the terminal differentiation of HL-60DM cells [13]. The drug-induced and, also, the spontaneous terminal differentiation of HL-60DM cells has been explained in terms of the exclusion of dmins from the cells by micronuclei [14]. By contrast, for some as yet unknown reasons, LEEs are not similarly excluded by micronuclei. The failure of micronuclei to exclude LEEs might explain why HL-60LEE cells are resistant to terminal differentiation [13, 15]. Therefore, it is of interest to define precisely the source of LEEs within the human genome and to identify the genes encoded by

individual LEEs. In the present study, we analyzed a library prepared from purified LEEs and, using fluorescence *in situ* hybridization (FISH) with human BAC clones as probes, we demonstrated that at least six segmented blocks, with five gaps, derived from a 4.4-Mb region of 8q24, are integrated into the LEE. We found at least four genes, including *NSMCE2* (for SUMO ligase), *TRIB1* (for a regulator of MAPK kinase), *LOC389687* (for a protein of unknown function) and *CCDC26* (for a retinoic acid-dependent modulator of myeloid differentiation), in addition to *Myc* within the 8q24 amplicon in the LEE. Moreover, the *NSMCE2* and *CCDC26* genes were interrupted by breaks in the amplified regions. We observed the constitutive expression of all five genes in HL-60 cells and, also, the suppression of the expression of four of them during the terminal differentiation of HL-60DM cells. FISH also revealed that one copy of chromosome 14 had changed to a small aberrant chromosome, der(14), in HL-60LEE cells. We show here that the genes encoded by the LEE are representative of genes whose expression should be suppressed during the terminal differentiation of HL-60 cells.

2. Materials and Methods

2.1 Cell culture, drug treatment, and the separation of differentiated from undifferentiated cells

The HL-60 line of human acute myeloblastic leukemia cells was obtained from the American Type Culture Collection (CCL240; ATCC, Manassas, VA, USA). The cells designated HL-60DM cells were early-passaged cells, examined prior to passage 28, that were confirmed to harbor dmins. HL-60LEE cells were late-passaged cells, examined after more than 150 passage; they contained LEEs but few dmins. All cells were grown in RPMI 1640 medium (Nissui, Tokyo, Japan), supplemented with 10% fetal calf serum (FCS). The absence of contamination by mycoplasma was confirmed. Induction of granulocytic differentiation by DMSO and the separation of differentiated from undifferentiated cells were performed as described previously [9]. In brief, cells were treated with 1.25% (v/v) DMSO (Nacalai tesque, Kyoto, Japan) for 48 h. Then the differentiated cells that had adhered to the substratum of the culture dish during a 1-h treatment with 100 nM phorbol 12,13-dibutyrate (Nacalai tesque) were carefully separated from non-adherent

undifferentiated cells that remained in suspension in the culture medium.

2.2. Chromosome sorting and analysis of the preparation of LEEs

Chromosomes were sorted as described elsewhere [16, 17]. A total of 10^8 cells was collected and suspended in 75 mM KCl after treatment with 0.03 μg/ml colcemid for 120 min. Cells were collected by centrifugation at 1,000 x g for 5 min and resuspended in 15 ml of polyamine buffer [15 mM Tris-HCl (pH 7.2), 20 mM NaCl, 80 mM KCl, 2 mM EDTA, 0.5 mM EGTA, 0.15% 2-mercaptoethanol, 0.2 mM spermine, 0.5 mM spermidine] and incubated on ice for 120 min. They were collected again and resuspended in 1.5 ml of polyamine buffer. Cells were lysed by addition of 1/9 volume of a saturated solution of digitonin (Sigma-Aldrich, St. Louis, MO, USA) in polyamine buffer, with immediate mixing on a vortex mixer three times for exactly 15 sec each. Intact cells and interphase nuclei were carefully removed by centrifugation at 50 x g for 5 min. The supernatant was obtained as the suspension of chromosomes. Then 5 μl of the suspension of chromosomes were mixed with 5 μl of a solution of propidium iodide (PI; 0.05 mg/ml in 0.1%

Triton X-100) and examined for contamination by nuclei and extraneous material by fluorescence microscopy. The suspension of chromosomes was stained with 75 μg/ml chromomycin A3 (Sigma-Aldrich) in 0.63 mM $MgSO_4$ at 4 ^{o}C for 12 h and then with 2.5 μg/ml Hoechst 33258 (Wako, Tokyo, Japan) for 4 h at 4 ^{o}C. The double-stained chromosomes in suspension were subjected to chromosome sorting in a FACS Vantage SE system (BD Bioscience, San Jose, CA, USA) that was equipped with two argon lasers (one was tuned for 457.9 nm excitation and the other was tuned for multiline UV excitation). Then, 1 μl of the sorted fraction, equivalent to 1,000 events, was dropped on a clean glass slide. After drying, the material was examined by FISH with the LSI-*MYC* probe (Vysis, Downers Grove, IL, USA).

2.3. Construction of a library from the LEE fraction

A DNA library was constructed from the LEE fraction by a previously reported method [18] with minor modifications. Sodium N-lauroyl sarcosine (1%; Sigma-Aldrich), EDTA (25 mM),

proteinase K (0.5 mg/ml) and 40 μg of glycogen (TAKARA, Tokyo, Japan) were added to the LEE fraction (400 μl). After gentle rocking at 50 ^{o}C for 16 h, proteinase K was inactivated by addition of 1/100 volume of 50 mM phenylmethylsulfonylfluoride (Sigma-Aldrich), with incubation at room temperature for 40 min. For partial digestion, a mixture of 4 units of Dam methylase (NEB, Ipswich, MA, USA) and 0.005 unit of *Mbo*1 (NEB) was added to 20 ng of ethanol-precipitated DNA in 20 μl of 30 mM Tris-HCl (pH 7.9), 50 mM potassium acetate, 9 mM magnesium acetate, 0.5 mM dithiothreitol, 80 μM S-adenosylmethionine and 0.03% bovine serum albumin, and the resultant mixture was incubated at 37 ^{o}C for 3 h. The DNA was dephosphorylated by incubation with 0.15 unit of calf intestinal alkaline phosphatase (TOYOBO, Kyoto, Japan) at 37 ^{o}C for 30 min, and the phosphatase reaction was stooped by incubation at 68 ^{o}C for 20 min with 15 mM nitrilotriacetic acid. From the resultant DNA, we prepared a library that contained 1.7 x 10^4 independent phage clones using the Lambda dash II vector (Stratagene, La Jolla, CA, USA) and Gigapack III Gold (Stratagene) according to the manufacturer's instruction. The nucleotide

sequence of the insert from each clone was determined with the MegaBACE™ 1000 DNA Analysis System (GE Healthcare Bio-Sciences, Uppsala, Sweden). The chromosomal loci of sequences were assigned by a BLAST search of the human genome (NCBI, Bethesda, MD, USA; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

2.4. Determination of copy numbers of STS (Sequence tagged site) sequences by real-time PCR

We quantified DNA by real-time PCR with a LightCycler™ system (Roche, Basel, Switzerland) and a Quantitect™ SYBR® Green PCR kit (QIAGEN, Valencia, USA). All reactions were performed in triplicate. The copy number of each STS sequence was determined by reference to the amount of the gene for ß-globin in human genomic DNA, as measured with the LightCycler™ control kit (Roche). All the sequences of STSs used for the determination of the copy numbers of genes in this study are shown in Supplementary Table 1.

2.5. Two-color FISH

DNA corresponding to various bacterial artificial chromosomes (BACPAC clones RP11-150N13, RP11-89K10, RP11-145G10, RP11-98N22, RP11-324B11, RP11-140M6, RP11-25B2, RP11-749G5, RP11-113A4 and RP11-48L1, which had been obtained from Children's Hospital Oakland Research Institute, Oakland, CA, USA; and BACPAC clones CTB-12C12, CTB-9N19, CTB-17K9 and CTD-2045M14 which had been obtained from Open Biosystems, Brussels, Belgium) was prepared with a Large-Construct kit (QIAGEN), as described in the instructions from QIAGEN. Biotin- and digoxigenin-labeled probes were prepared with a BioPrime^{TM} kit (Invitrogen, Carlsbad, CA, USA) and a Dig-High Prime^{TM} kit (Roche), respectively, according to the protocols from the respective manufacturer. The preparation of slides and FISH were performed as described elsewhere [13, 19]. The material on the slides was denatured in 70% formamide in 2x SSC (SSC is 15 mM sodium citrate, 150 mM NaCl) at 70 ^{o}C for 2 min and washed successively in 70%, 85% and 100% ethanol for 5 min each. Ten microliters of hybridization

solution (2x SSC with 10% dextran and 40% formamide) that contained 2 μg of human cot-1 DNA (Invitrogen), 2 μl of biotin-labeled probe and 2 μl of digoxigenin-labeled probe were heated at 76 ^{o}C for 10 min to denature DNA, chilled on ice for 1 min and applied to the target area of the slide. The material on the slide was then covered with a glass coverslip and hybridization was allowed to proceed at 37 ^{o}C for 16 to 24 h in a humidified box. After hybridization, each slides was washed three times in 2x SSC that contained 50% formamide and once in 2x SSC that contained 0.1% NP-40. For the detection of signals, the target area was incubated at 37 ^{o}C for 20 min with blocking buffer [4x SSC plus 5% Blockace^{TM} (Dainippon Pharm, Osaka, Japan)] and for 40 min with 100 μg/ml streptavidin-fluorescein isothiocyanate (Roche) and 4 μg/ml digoxigenin-specific antibodies raised in sheep (Roche), in blocking buffer. When we used the LSI-*MYC* probe (Vysis) instead of a digoxigenin-labeled probe, we omitted the digoxigenin-specific antibodies. The slides were then washed three times for 10 min each in 4x SSC with 0.05% Tween-20 and counterstained with DAPI II (Vysis), which contained 1 mg/ml 4,6-diamidino-2-phenylindole (Sigma-Aldrich) as

an antifade reagent. Images were recorded with an Aquacosmos/VIM Microscope System (Hamamatsu Photonics, Hamamatsu, Japan).

2.6. Quantification of gene expression by real-time RT-PCR

Total RNA was purified with the Versagene™ RNA purification system (Gentra Systems, Minneapolis, MN, USA). Target genes for quantification of levels of expression were selected by reference to the NIH map of the human genome (NCBI; http://www.ncbi.nlm.nih.gov/mapview/). The primer sequences for RT-PCR were designed with the aid of the Primer3 program [20]. The sequences of primers that we used for quantification by RT-PCR were as follows: 5'-AAGCTGACGGAACAGAAGGA-3' and 5'-CTCAATCATGCGAACAATGG-3' for *NSMCE2*; 5'-ACTACCTGCTGCTGCCCCTA-3' and 5'-GGCCTGATTTTGTCCTGGTA-3' for *TRIB1*; 5'-TCTTCCAGATATCCTCGCTG-3' and 5'-TATGACCTCGACTACGACTCG-3' for *Myc*; 5'-AAAGGACCATGGGCTGTTTG-3' and 5'-CATTTGGTAATTCTAGGTGATCTGG-3'

for *CCDC26*; and 5’-TAGTGAGGCCCCAAATCAAA-3’ and 5’-AGCAGGGTCTGGACATGAAG-3’ for *LOC389687*. Quantification of each mRNA was performed with the Quantitect™ SYBR® Green RT-PCR kit and LightCycler™ system. The level of expression of each gene was standardized by reference to that of the gene for ß-actin in each culture. All products of RT-PCR were confirmed by monitoring electrophoretic mobility and by sequence analysis.

2.7. Analysis by RACE

Analysis by 3’ RACE (rapid amplification of cDNA ends) of *CCDC26* was performed with Gene Racer™ (Invitrogen) according to the protocol from the manufacturer. We used the oligonucleotide 5’-TGGGCTGTTTGAGGGGCCAACATCA- 3’ as the *CCDC26*-specific primer.

3. Results

3.1. Purification of LEEs by chromosome sorting

We prepared a suspension of chromosomes from HL-60LEE cells with special care, in an effort to avoid breaking the chromosomes. The chromosomes of HL-60LEE cells were more fragile than those of peripheral lymphoid cells and, for example, Daudi cells (data not shown). We monitored and took care to maintain a clearly distinguishable pattern of all chromosomes, including numbers 1 through 22 and chromosome X, during the sorting and manipulation of chromosomes (Fig. 1a). We collected a fraction with lower fluorescence than that of the smallest human chromosomes, namely, chromosomes 21 and 22. We also excluded submicroscopic extrachromosomal DNA by controlling the collection gate [19, 21]. The fragility of the chromosomes from HL-60 cells resulted in some contamination by fragments of broken chromosomes in the collected fraction. To evaluate the purity of this fraction, we fixed samples on slides and examined the results of staining with the LSI-*MYC* probe. We found that 40% (80/200) of DAPI-stained structures were LEEs that

hybridized with the LSI-*MYC* probe (Fig. 1b). By contrast, 90% of DAPI-stained structures in a fraction that contained chromosome 8 and had been prepared as a control hybridized with the same probe (Fig. 1c).

3.2 Analysis of a DNA library prepared from the LEE fraction

We generated a shotgun library from DNA that we had extracted from the purified LEE fraction, which was prepared as described above. The results of the sequence analysis of 183 clones that we picked at random from this library are summarized in Figure 2a. The distribution of clones revealed many irrelevant clones that corresponded to sequences scattered over all the chromosomes, and these latter clones were probably derived from broken chromosomes. By contrast, 40 of the clones represented sequences that were concentrated at two loci, namely, 8q24, which includes *Myc* (Fig. 2b), and 14q11 (Fig. 2c). The region to which 23 of these clones could be assigned on 8q24.12-.21 extended for 4.4 Mb, with a proximal breakpoint (pb) at 126,311,010 and a distal

breakpoint (db) at 130,735,870 (the numbering is based on NCBI mapviewer *Homo Sapiens* Built 35.1), and this region includes the *Myc* gene (128,817,686-128,822,856). The region to which the remaining 17 clones could be assigned, on 14q11.1-.2, extended for 8.4 Mb (18,583,850-26,944,590) and includes genes for T-cell receptors (21,159,897-22,090,923). No more than three clones could be assigned to any other individual locus.

3.3. Mapping of the amplicon with STS (sequence tagged site) sequences

To map the amplified region that corresponded to the LEEs, we chose STSs that had been registered at 8q24 and 14q11 on the map of the human genome [22] and determined the copy numbers of the various sites by quantitative real-time PCR (Figs. 2b and 2c). We detected four to twenty copies of STSs relative to the copy number of the gene for ß-globin in the region between SHGC-56877 (121,263,776) and the *CCDC26* gene (130,761,666). We determined that there were eight copies of the *Myc* gene in HL-60LEE cells and this number was consistent with that

measured previously by competitive PCR [9, 23]. We also found at least five gaps (Fig. 2b), for which no increase of the copy number (less than two fold) of STSs was detected, in the 4.4-Mb region of 8q24. Thus, the amplicon unit of the LEEs had a discoutinuous structure, including at least six regions of 8q24 (designated Amp 1 through Amp 6). By contrast, we failed to detect any significant increase of copy number (more than two fold) of STSs in the region of 14q11, in which the sequences of 17 clones in the LEE library were concentrated, as noted above (Fig. 2c).

3.4. Analysis by FISH of 8q24 and 14q11 loci

We performed FISH using, as probes, BAC clones of sequences located within 8q24 and 14q11. RP11-150N13 was located near the pb, while CTB-17K9 and CTB-9N19 were located near the db in the amplicon of 8q24 (Fig. 2b). LSI-MYC is a commercially available probe that corresponds to a region of 150 kb in the vicinity of the *Myc* gene (Fig. 2b). Each of these probes hybridized with the LEEs and with the dmins, indicating that sequences near both the pb and the db had been

co-amplified with *Myc* in both HL-60LEE and HL-60DM cells (Figs. 3a-3e). By contrast, the additional 8q24-specific BAC probes RP11-89K10, RP11-145G10 and CTB-12C12, which corresponded to gaps in the amplified region, hybridized to neither the LEEs nor the dmins (Fig. 3f for CTB-12C12; data for RP11-89K10 and RP11-145G10 not shown). One of these probes, CTB-12C12, hybridized with dmins from Colo320DM cells [24], whereas CTB-17K9 did not (Fig. 3g). These observations and our unpublished results indicate that the amplicon in Colo320 cells have a continuous structure different from that in HL-60 cells.

FISH with several BAC probes, including RP11-140M6, RP11-98N22, RP11-324B11 and CTD-2045M14, which were specific for 14q11, gave essentially the same results: each probe hybridized with one chromosome 14 and one small chromosome in HL-60LEE cells (Figs. 4a-4c; data for RP11-324B11 not shown). The RP11-140M6 probe also hybridized to 22q11.1, a result that is not surprising since this chromosomal region includes highly repetitive sequences (Fig. 4b). The length of the small chromosome was 30 to 50% of that of chromosome 22 and the small

chromosome was slightly larger than the LEEs (Figs. 4a and 4b). By contrast, RP11-45L1, which corresponded to 14q12, hybridized to only one chromosome, which retained its original size, and to no others in HL-60LEE cells (Fig. 4c). Moreover, all of the probes specific to chromosome 14 hybridized to two chromosomes of equivalent size in HL-60DM cells (Fig. 4d for RP11-45L1; data for other probes not shown). These results indicated that, in HL-60LEE cells, one copy of chromosome 14 had been truncated between 14q11 and 14q12 to yield a novel derivative of chromosome 14 [der(14)], with a terminal deletion (Fig. 4e).

3.5. Expression of genes within the amplicon

To quantify the expression of genes in the amplified regions, we performed quantitative real-time RT-PCR using primer pairs that corresponded to the sequences of the respective mRNAs. We used a culture of HL-60DM cells that had been treated with 1.25% DMSO and had been separated into irreversibly differentiated cells with a lower number of copies of *Myc* (an average of four

copies) and undifferentiated cells with a higher number (an average of 39 copies [9]). We used DMSO-treated but unfractionated HL-60DM cells as a control for the effects of the phorbol ester that had been used for the fractionation of cell cultures. We detected constitutive expression of five genes in the amplicon of the 8q24 locus, namely, *NSMCE2, TRIB1, Myc, CCDC26* and *LOC389687,* in HL-60 cells. We calculated and compared the relative levels of expression of each gene in undifferentiated and differentiated HL-60DM cells (Fig. 5). The differences in relative levels of expression (undifferentiated/differentiated cells) of the *Myc*, *NSMCE2, TRIB1*, and *LOC389687* genes were approximately 16-fold, 20-fold, 4-fold and 26-fold, respectively. The level of expression of *CCDC26* in differentiated cells was too low to allow calculation of a ratio in this system, but we estimated that the difference was more than 10-fold. We detected a significantly higher level of expression of *TRIB1* in both differentiated and undifferentiated cells than in non-fractionated cells, probably as a result of the exposure of cells to the phorbol ester that had been used during fractionation of cell cultures [25].

3.6. Determination by RACE of the 3' terminus of CCDC26

The break in the *CCDC26* gene was located between the regions Amp 5 and Amp 6 and, therefore, we performed RACE to identify the 3'-terminus of this gene. We detected several variants of mRNAs that differed from the authentic product of this gene. Three of them are shown schematically, with the authentic mRNA, in Figure 6c. The variants corresponded to various different regions of the amplicon, including Amp 5, Amp 6 and Amp 2. No abnormalities in *CCDC26* were detected in U937 cells, in which no chromosomal amplification of 8q24 occurs ([26] and our unpublished result).

4. Discussion

The breakpoints in the 4.4-Mb amplicon of HL-60 cells that we identified in the present study are consistent with the cluster regions of pbs (126,000,000-126,500,000) and dbs (130,800,000-131,400,000) in dmins in recently described cases of acute myeloid leukemia and myelodysplastic syndrome [27,28]. Furthermore, we found at least five gaps in the amplicon in HL-60 cells. The location of the gap that we identified between Amp 4 and Amp 5 is consistent with that in an earlier report about the HL-60 amplicon [29]. The complicated structure of the amplicon suggests that multiple events are involved in its generation in HL-60 cells [3,4]. Discontinuity in amplicons has been observed in other cancer cells, including neuroblastoma and glioma cells, and was considered to be the result of excision of a preceding larger circular amplicon [30, 31, 32]. Our observations imply, however, that nascent replication rather than random deletion is involved in the generation of the discontinuous amplicon. The structure of the amplicon, which consists of segments clustered in a restricted chromosomal region (126.2 Mb to

137.6 Mb) with interruption of 0.2 to 1.2 Mb, is reminiscent of replication bubbles that form from multiple initiation points within a replication unit at the early stage of chromosomal replication [33]. Indeed, one of the major sites of initiation of DNA replication is reported to be located at the *Myc* locus [34]. Figure 7 depicts a possible model that explains the generation and development of the amplicon. In this model, several replication bubbles form as a result of stalled elongation at the replication fork in the 8q24 chromosomal region; these bubbles collapse and the "corrupted" bubbles are rejoined by a repair mechanism to generate an initial amplicon composed of Amp units (numbered 1 through 6 here). Thereafter, the amplicon might be converted to larger structures, such as a dmin (by duplication) and a LEE (by multimerization). Although either inverted or tandem repeats can result in duplication, a tandem-repeat structure seems more plausible from two-color FISH images of dmins (Fig. 3b and 3c). Assuming that the complete amplicon unit is an array of the amplified regions Amp 1 through Amp 6, as indicated by the units 1 through 6 in Figure 7, we can calculate that the entire amplicon unit is 0.9 to 1.0 Mb in length. Since the length

of dmins in early-passaged HL-60 cells was estimated by Von Hoff *et al*. [35] to be 1.5 to 3.0 Mb, each dmin should include two or three repeats of the amplicon unit itself. By contrast, we estimated that each LEE was approximately 12 Mb long (25% of the length of chromosome 21). Thus, each LEE should include 10 to 15 repeats of the amplicon. Our two-color FISH images of dmins and LEEs, recorded after hybridization with probes for two different regions of 8q24, support these estimations (Figs. 3a-3e). No structural differences between the amplicon units of LEEs and dmins have been observed to date.

The clones that corresponded to 14q11 in the LEE shotgun library appeared to originate from a small aberrant chromosome, der(14). It is likely that LEEs and der(14) were co-purified during the sorting of chromosomes. We only detected der(14) in HL-60LEE cells and we never found it in HL-60DM cells. During the conversion of HL-60DM cells to HL-60LEE cells, one copy of chromosome 14 probably underwent terminal deletion at 14q11-12. One copy of chromosome 14 in early-passaged HL-60 cells was found to have an abnormal q arm in earlier karyotypic analyses

[36, 37]. These observations suggest the presence of a fragile region in 14q in HL-60 cells (Fig.4e). The conversion of chromosome 14 to der(14) during the progression from HL-60DM to HL-60LEE cells has not previously been reported, to our knowledge. It is possible that the region that includes 14q12 and is absent from HL-60LEE cells might encompass a gene(s) whose absence contributes to the resistance of these cells to apoptosis. However, we have no evidence for any specific roles of genes on chromosome 14 in HL-60 cells during the progression of HL-60DM to HL-60LEE cells at the present time.

Detailed mapping of the amplicon unit revealed abnormalities in the structures of two large genes (Figs. 6a and 6b). First, since the pb of the amplicon unit on chromosome 8 was located between exon 5 and exon 6 of the *NSMCE2* gene, only the carboxy-terminal coding region of the *NSMCE2* gene was present in the amplicon unit (Fig. 6a). Second, only exon 1 through exon 3 of the *CCDC26* gene was present in the Amp 6 region and exon 4 was not found in the amplicon unit (Fig. 6b). These observations indicate that these two genes are sources of aberrantly truncated products

in HL-60 cells. Abnormalities in the *NSMCE2* gene might be associated with the malignancy of leukemia cells since truncation of this gene has been demonstrated in some cases of acute myeloid leukemia and of myelodysplastic syndromes with dmins [28].

We considered the possibility that some gene(s) within the LEE, other than *Myc*, might be responsible for the resistance of HL-60LEE cells to terminal differentiation and apoptosis since expression of *Myc* in HL-60 cells that are treated with DMSO is suppressed prior to terminal differentiation [11]. Any candidates for such a gene must be constitutively expressed in HL-60 cells and their expression must be repressed during terminal differentiation since HL-60DM cells remain undifferentiated until such genes are excluded by micronucleation [13]. *LOC389637*, a gene that is amplified with *Myc*, is expressed at a very low level during terminal differentiation. Therefore, this gene is a candidate for a gene that might be responsible for the terminal differentiation of HL-60 cells. Unfortunately, this gene is a putative gene of unknown function so no conclusions about its role can be drawn at present. The levels of expression of *NSMCE2* in

undifferentiated and differentiated cells were very different. The *NSMCE2* gene encodes a SUMO ligase, and inhibition of the activity of this ligase in HeLa cells results in apoptosis [38]. It is possible that an abnormal truncated protein, encoded by *NSMCE2,* might be produced from the amplified genes and might be responsible for the resistance of HL-60LEE cells to apoptosis. *CCDC26,* whose expression was not detected in differentiated cells, is also a candidate for a gene that might be responsible for the resistance of HL-60LEE cells to terminal differentiation. In a recent study, retrovirus-mediated insertional mutagenesis of this gene between exon 1 and exon 2 was detected in a drug-resistant subline of HL-60 cells [39]. The expression of *TRIB1* was clearly induced by the phorbol ester that we used to fractionate our cultures and this gene might also play a role in the malignancy of HL-60 cells. The *TRIB1* gene encodes a modulator of the activity of a MAPK kinase with several physiological functions [40], whose expression has been detected in lines of tumor and tumor-derived cells [25, 27, 28].

Unlike dmins, LEEs appear to be very stable and are not excluded by drugs that promote the

formation of micronuclei [13]. A possible explanation for the stability of LEEs might be the integration of a stability-related *cis*-element during the evolution of LEEs. To date, however, we have found no sequences in LEEs that are not also present in dmins. Another possible explanation is the acquisition of a functional centromere, which would stabilize abnormal chromosomes in the absence of alphoid sequences by epigenetic mechanisms that include methylation of DNA, acetylation and phosphorylation of histones, and the poly(ADP-ribosyl)ation and ubiquitination of centromere proteins [41].

In summary, the expression of genes encoded by LEEs is suppressed during the terminal differentiation of HL-60DM cells. These genes might be candidates for the gene(s) that is responsible for the resistance of HL-60LEE cells to terminal differentiation. Therefore, LEEs, which include amplified genes whose products maintain the undifferentiated state of HL-60 cells, might have evolved selectively as more stable extrachromosomal elements than dmins.

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Legends to Figures

Figure 1. (a) Karyogram of the chromosomes of HL-60LEE cells, after sorting by flow cytometry. The sorted and collected area that corresponds to LEEs is indicated by a red polygon. (b) Results of FISH of the sorted LEEs with the LSI-*MYC* probe. Two of the four blue DAPI-stained structures hybridized with the LSI-*MYC* probe. Arrows indicates LEEs. (c) Results of FISH of sorted chromosome 8, as a control. The 8q24 locus of two sorted chromosomes that hybridized with the LSI-*MYC* probe is indicated by arrows. Bars, 10 μm.

Figure 2. (a) Genomic mapping of clones in the LEE library to all the chromosomes in the human genome by comparison of sequences. Each clone is designated by a red dot beside a chromosome. Thirteen clones could not be mapped because they contained highly repetitive sequences. (b) Detailed mapping of 23 clones in the 8q24 chromosomal region. The copy numbers of STSs are indicated on the histogram (Y axis). The locations of clones (indicated by

red pins), BAC probes (horizontal lines beneath the histogram) and genes (vertical bars and horizontal lines) are shown. *LOC389687* was located within an intron of *CCDC26*. The locations of the proximal breakpoint (pb) and the distal breakpoint (db) are indicated. The zones labeled "Amp 1" through "Amp 6" represent the amplified regions within the amplicon. The asterisk next to a pin below the histogram indicates a clone with the sequence of 128,838,800 in Amp 4 at one end and that of 130,081,730 in Amp 5 at the other end, with a gap. Cen and Tel with arrows indicate the directions of the centromere and the telomere, respectively. (c) Detailed mapping of 17 clones in the region at 14q11-14q12. The locations of clones (pins) and BAC probes (horizontal lines beneath the histogram) are shown. The directions of the centromere and the telomere are also indicated. The chromosomal range of der(14) (see also Fig. 4) is indicated.

Figure 3. Examples of images obtained by FISH of 8q24. The following probes were used: CTB-9N19 (green) and LSI-*MYC* (red) in (a) through (d); RP11-150N13 (green) and LSI-*MYC*

(red) in (e); and CTB-12C12 (green) and CTB-17K9 (red) in (f) and (g). (a) Metaphase chromosomes and the interphase nucleus of an HL-60LEE cell. Arrows and arrowheads indicate LEEs and chromosome 8, respectively. (b) The metaphase chromosomes of an HL-60DM cell. dmins are visible in the inter-chromosomal space. (c) The interphase nucleus of an HL-60DM cell, in which amplified DNA was detected. (d) The interphase nucleus of an HL-60DM cell in which micronucleation was observed (arrow). (e) The metaphase chromosomes of an HL-60LEE cells. Arrows indicate LEEs. (f) Metaphase chromosomes and the interphase nucleus of an HL-60LEE cell. Arrows and arrowheads indicate LEEs and chromosome 8, respectively. (g) The metaphase chromosomes of a Colo320DM cell. CTB-12C12 (green) signals were observed on dmins, whereas CTB-17K9 (red) signals (arrowheads) were not. Bars, 10 μm.

Figure 4. Examples of images obtained by FISH of 14q11. The following probes were used: CTD-2045M14 (green), specific for 14q11, and CTB-9N19 (red), specific for 8q24, in (a);

RP11-98N22 (green) and RP11-140M6 (red), both specific for 14q11, in (b); and CTD-2045M14 (green), specific for 14q11, and RP11-48L1 (red), specific for 14q12, in (c) and (d). (a) The metaphase chromosomes of an HL-60LEE cell. The white arrow and asterisk (*) indicate chromosome 14 and der(14), respectively. Red arrows and arrowheads indicate LEEs and the 8q24 locus, respectively. (b) The metaphase chromosomes of an HL-60LEE cell. The arrow and asterisk (*) correspond to those in (a). The arrowhead indicates chromosome 22. (c) The metaphase chromosomes of an HL-60LEE cell. The arrow and asterisk (*) correspond to those in (a). The RP11-48L1 (14q12; red) probe did not stain der(14). (d) The metaphase chromosomes of an HL-60DM cell. The arrows indicate two chromosomes 14 of similar size. (e) Summary of the difference in status of chromosome 14 between early- and late-passaged HL-60 cells. Bars, 10 μm.

Figure 5. Results of quantitative RT-PCR. Levels of expression of *Myc*, *NSMCE2*, *TRIB1*,

LOC389687 and *CCDC26* were normalized by reference to the level of expression of the gene for ß-actin. The histograms show relative levels of expression of each gene in HL-60LEE cells (HL-60; values for these cells were set to 1), in differentiated HL-60DM cells (Diff), in undifferentiated HL-60DM cells (Undiff) and in HL-60DM cells that were treated with DMSO but not fractionated (DMSO), on a logarithmic scale. For each gene, the size of the product of RT-PCR (arrowhead) was determined by electrophoresis in a 1.8% agarose gel. Lanes on gels correspond to the order of columns in the histograms, excluding the marker lane (far left) and the lane that corresponded to RT-PCR without RNA (far right). Approximate ratios of levels of expression are also shown. No product of RT-PCR corresponding to the expression of *CCDC26* in differentiated cells was detected (*).

Figure 6.

Detailed maps of the amplicon regions of (a) Amp 1, including *NSMCE2*

(126,173,277-126,4448,544); and (b) Amp 6, including *CCDC26* (130,433,122-130,761,667). The horizontal lines and vertical bars indicate genes and exons that correspond to *NSMCE2* and to *CCDC26*. The numbering of exons was deduced by aligning cDNA sequences (1,258 bp, NCBI accession number NM_173685 for *NSMCE2*; and 1,750 bp, NCBI accession number NM_145050 for *CCDC26*) with genomic sequences. Amplification of exons 6 through 8 in *NSMCE2* and of exons 1 through 3 in *CCDC26* was detected (indicated by "+" signs above histogram). Locations of pb, db, and copy numbers of STSs are as in Fig. 2.

(c) Authentic and non-authentic mRNA variants derived from *CCDC26*. The top bar indicates the authentic mRNA and other bars indicate mRNA variants detected by 3' RACE. The black box indicates the coding region of the *CCDC26* gene. Numbers (130,451,805 and 130,562,069) correspond to junctions of exons 2 and 3 of the gene. Unshaded regions in mRNA variants were also found in the authentic mRNA. Hatched and gray bars in variants indicate parts of regions from Amp 6 (130,481,114-130,480,738 and 130,766,988-130,766,829) and Amp 2

(126,826,336-126,826,441), respectively. Dotted bars, namely, the region from Amp 5 (130,183,817-130,183,719), are identical for two of the variants. Arrows indicate primers used to detect variant mRNAs. The primer located in the common region was 5'-GAGGGGCCAACATCATTAAC-3' and primers in the regions specific to variants were 5'-ACAGGGTCCCTCCACAACAT-3' and 5'-CTCATGAATTGAAGGGACATCA-3'. Results of analysis by RT-PCR of non-authentic variants with each specific primer are shown with sizes of products (171 b and 122 b) in the right panel. Excessive PCR produced non-specific background smears in the lane designated U937.

Figure 7

A model for the generation of the discontinuous amplicon unit of dmins and LEEs in HL-60 cells. Several replication initiation bubbles collapse and “corrupted” bubbles rejoin to form an amplicon unit. Excision of an initial large amplicon (as an episome?) might perhaps, precede replication.

The arrows numbered 1 through 6 indicate regions Amp 1 through Amp 6 (see Fig. 2). Note that the lengths of the various regions are not to scale. Once the amplicon unit has formed, its multimerization results in larger structures that include dmins and LEEs.

Figure1

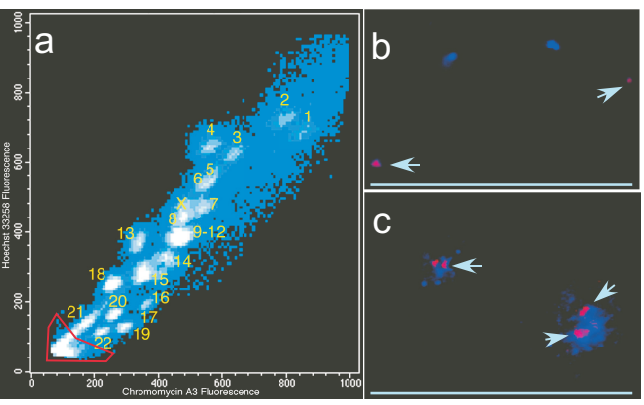
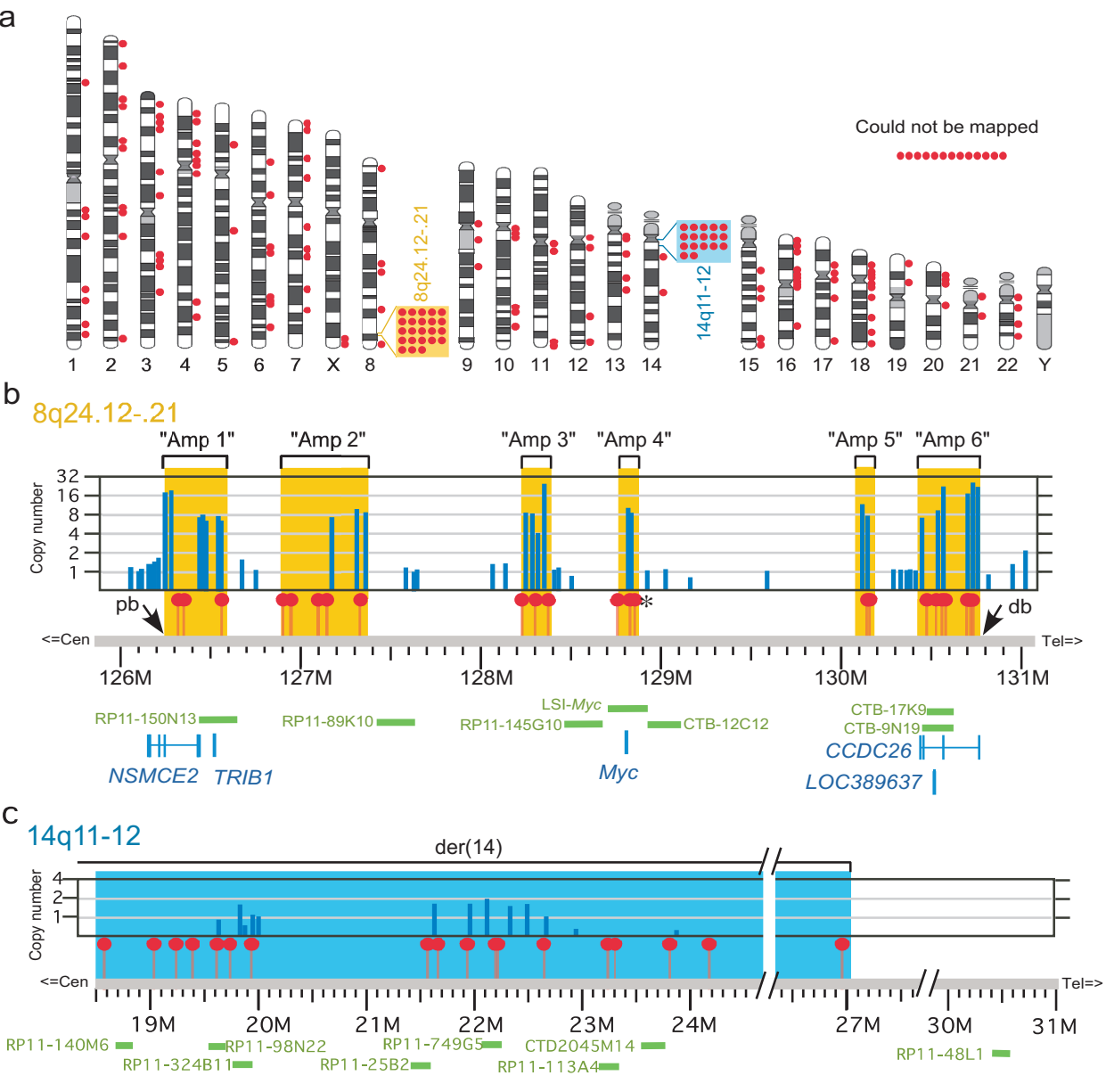

a
b
c
Hoechst 33258 Fluorescence
Chromomycin A3 Fluorescence
0
200
400
600
800
1000
1
2
3
4
5
6
7
8
9-12
13
14
15
16
17
18
19
20
21
22

Figure2


a
Could not be mapped
8q24.12-.21
14q11-12
1 2 3 4 5 6 7 X 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 Y
b
8q24.12-.21
"Amp 1" "Amp 2" "Amp 3" "Amp 4" "Amp 5" "Amp 6"
Copy number
32
16
8
4
2
1
pb
db
<=Cen
Tel=>
126M 127M 128M 129M 130M 131M
RP11-150N13
RP11-89K10
LSI-Myc
RP11-145G10
CTB-12C12
CTB-17K9
CTB-9N19
NSMCE2
TRIB1
Myc
CCDC26
LOC389637
c
14q11-12
der(14)
Copy number
4
2
1
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Tel=>
19M 20M 21M 22M 23M 24M 27M 30M 31M
RP11-140M6
RP11-324B11
RP11-98N22
RP11-25B2
RP11-749G5
RP11-113A4
CTD2045M14
RP11-48L1

Figure3

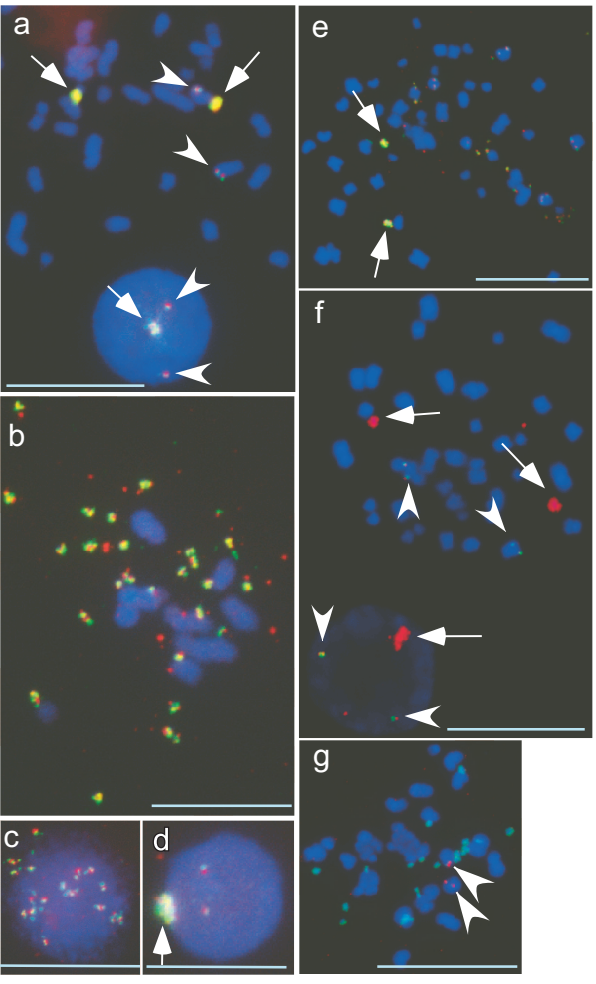
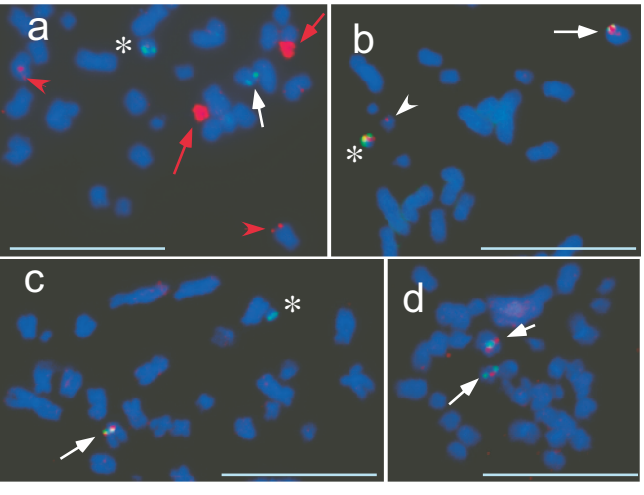


Figure4


a
b
c
d

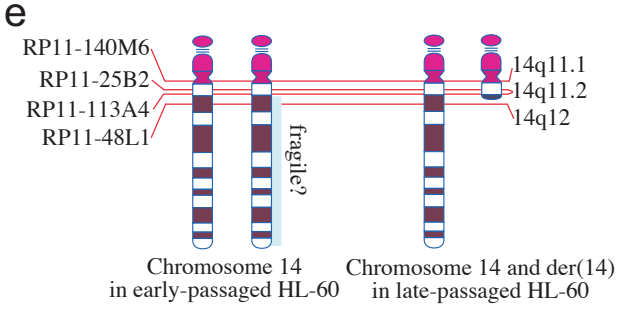
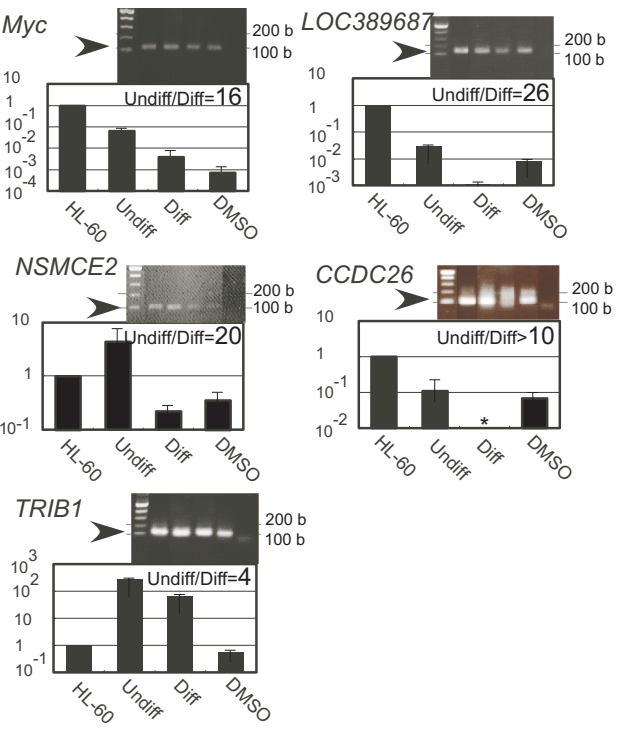

e
RP11-140M6
RP11-25B2
RP11-113A4
RP11-48L1
fragile?
14q11.1
14q11.2
14q12
Chromosome 14
in early-passaged HL-60
Chromosome 14 and der(14)
in late-passaged HL-60

Figure5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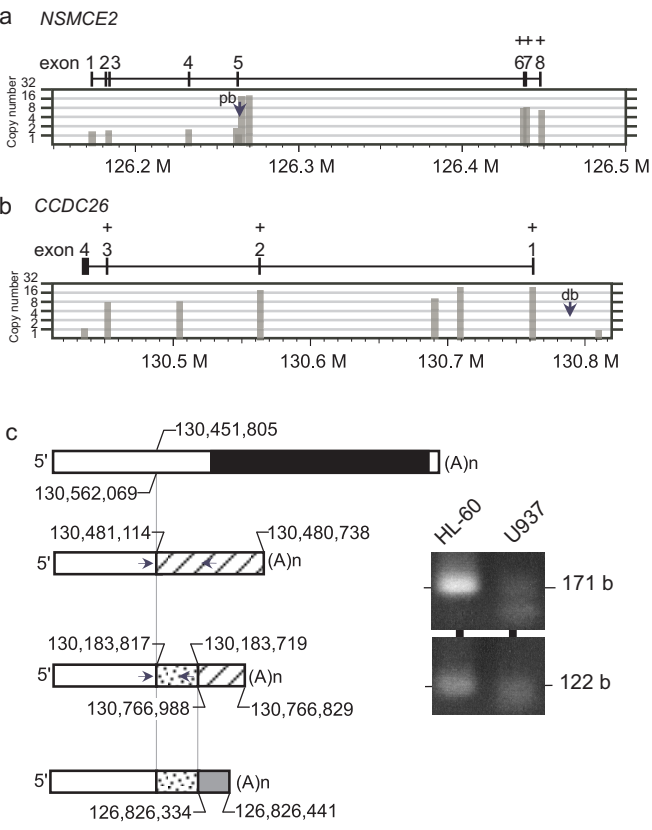
100 b
Undiff/Diff=16
HL-60
Undiff
Diff
DMSO
LOC389687
200 b
100 b
Undiff/Diff=26
HL-60
Undiff
Diff
DMSO
NSMCE2
200 b
100 b
Undiff/Diff=20
HL-60
Undiff
Diff
DMSO
CCDC26
200 b
100 b
Undiff/Diff>10
HL-60
Undiff
Diff
DMSO
TRIB1
200 b
100 b
Undiff/Diff=4
HL-60
Undiff
Diff
DMSO

Figure6


a
NSMCE2
exon 1 2 3 4 5 6 7 8
Copy number
32
16
8
4
2
1
pb
126.2 M
126.3 M
126.4 M
126.5 M
b
CCDC26
exon 4 3 2 1
db
130.5 M
130.6 M
130.7 M
130.8 M
c
130,451,805
5'
(A)n
130,562,069
130,481,114
130,480,738
130,183,817
130,183,719
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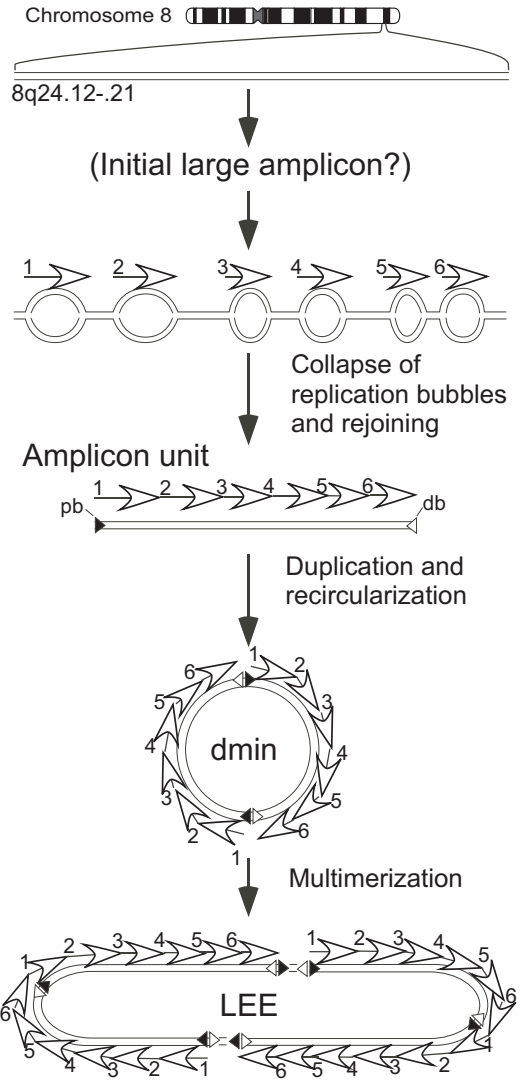
130,766,829
126,826,334
126,826,441
HL-60
U937
171 b
122 b

Figure7


Chromosome 8
8q24.12-.21
(Initial large amplicon?)
1
2
3
4
5
6
Collapse of
replication bubbles
and rejoining
Amplicon unit
pb
db
Duplication and
recircularization
dmin
Multimerization
LEE

Supplementary Table. List of the STS primers used in quantitative PCR

STS site	Locus	Begins at	Ends at	forward primer	reverse primer
WI-5950	8q24	126,059,555	126,059,762	GTGACTAAAAATGCATTTGAAAAA	CCTGAGAGTAAGCAAAGGAACC
D8S1682	8q24	126,103,472	126,103,643	CTGTAGTTCTCCTCAGCATT	GTGGAAACTCTTGGACCAAG
G20790;A006G23	8q24	126,105,701	126,105,901	CTATTTTACTGAAAATCTGGAGT	AGTAAGATATATCTCATGGCATT
D8S1483	8q24	126,156,531	126,156,657	ATTACCTGAAAGAGGAGACATTCA	TGGTTTTTCATTGGAATGGA
FLJ32440_EFg	8q24	126,173,361	126,173,516	CGGAGACAGCTTGGACTACC	CTACCGTGGCGTGAAAAGTT
FLJ32440_GHg	8q24	126,183,481	126,183,587	GCACTCTTTTGGAAGAGGGTAA	ATCACTGTGCCACTCACCTG
FLJ32440_IJg	8q24	126,183,748	126,183,902	TTTCAAGATGCCAGGACGTT	CCACAAGATCCAAAGCAACA
D8S371	8q24	126,210,946	126,211,189	AGTGTTAGGAAAAGATTTGGC	AATGAGGTGAAAGAAACTGCA
SHGC-154637	8q24	126,211,502	126,211,849	ACGCTTCTTAGTTGGTTTCGCTT	TTGAGTGTGGATACAGCGAGCTA
FLJ32440_KLg	8q24	126,232,624	126,232,888	AAGGCAATGGTTGAATTTGC	GTGGCCTAGCCAGGAAAATA
FLJ32440_MNg	8q24	126,263,519	126,263,807	TCCCTTAGGTGAAAGAAGAACG	CGCAACTTAGAATGAGGATCAA
SHGC-56887	8q24	126,263,776	126,263,963	GTTTTCCCAATTTCCCTCCC	TCTGCTTGGCTTGATCCTCA
RH11704	8q24	126,320,746	126,320,940	ATGTCTCAACCACCTGAGTGG	AATTGGGTGGGGCACTGT
FLJ32440_OPg	8q24	126,438,664	126,438,797	AAGCTGACGGAACAGAAGGA	TGCCTCTGTGAAGTGAATCG
FLJ32440_QRg	8q24	126,439,102	126,439,213	CATTCCTGAAAGCCCAACAT	CTCAATCATGCGAACAATGG
A009A17	8q24	126,448,272	126,448,479	CTGTTGTGTAACTTTGAAAAATG	AGAACCATAACAAGAAAAGACAT
G29269	8q24	126,553,184	126,553,333	ACTGACAGTGCCAACTGATGATG	GCCAACAAATTGGGATTATCTCAAC
G34243	8q24	126,553,952	126,554,048	TGTGCATGTTATCTGACTGATCC	GCCTTCAGGGTTGCTTATGA
G32128	8q24	126,670,596	126,670,732	ACACAGCGGGTCTTCTTGAT	GGGAGACCATGTTTGCAGAT
G32205	8q24	126,766,623	126,766,799	CATGTTCACCCTCATCATGC	GGCCCCTAAATAGATCCCTG
D8S1519	8q24	126,931,304	126,931,681	GCATGAGCCACCACACCTG	AGGCCTGCTCAGTTCAGGG
WI-10867	8q24	127,146,742	127,146,969	TTGTGCTCTTTACTGTTGTTTGTAT	CAAATGAGCAAACAAAATACCTT
SHGC-151078	8q24	127,305,963	127,306,156	GCTCACTGAGTGGCCAATTTTA	AGCTCTGGCAAGTTGAAGGAAT
D8S1793	8q24	127,374,809	127,374,951	TGAGCCGAGTTCTTACCAC	AACAAGTCCAGCTTGATGAG
RH103446	8q24	127,581,431	127,581,616	GAGCTCTTCCCAGCCTTTG	TGTTTTGGTTTTGTTTTACCCC
RH103480	8q24	127,634,721	127,634,854	CTTCAGAAGTTCTGGCAGGG	GTTGTGGGCTCCTTTGACAT
RH44809	8q24	127,638,064	127,638,314	ACGATCTGTACCGCTACA	GATCAGGTCCTCTAGACTCTG
G10165	8q24	128,073,628	128,073,801	TTTATCTACCCAACCACCCA	TATGTCTGCCTTTCCTGGAG
STS-H98688	8q24	128,135,772	128,135,957	AGGGTCGATGTCCATTAAGG	CGACTTAGAATGAGGTTCGG
RH81049	8q24	128,218,335	128,218,548	TCACATTTATCATTGCTGTACCA	TGCTTTCTTCCTTTCATTCCA
RH48146	8q24	128,286,272	128,286,394	GATAGCTGTAAGGAGAGTGGGC	CCTGTGTACACCTGTGTAACCA
G59703	8q24	128,310,330	128,310,520	TGACTTGACGAAGCTCATAGAAGA	CTAGATCGTGTGCTTGCCAGAAT
SHGC-141909	8q24	128,379,683	128,379,997	CATGGGTTTCCTCCCTCTCTAAT	GAACACCATCACCCTGAGTTTTC
D8S1090	8q24	128,423,616	128,423,795	TTCAGAGCACAGGGCTTATT	TAAGCAAGTATTTGGCAGGG
RH48719	8q24	128,435,399	128,435,574	CCCTGTAAAGTCTGCTTTCACC	AGGAAGTCTTCTTCCCTTGAGG
RH77966	8q24	128,498,556	128,498,745	TGCTCTTGATTTTATTCCCACA	GGTAAAAAGGAGGAAAAAAGCA
SHGC-6067	8q24	128,612,493	128,612,632	ATTTGAACTGAGTCTTGGCA	CTTTTCTCTGAACCAAGCTTC
G15987	8q24	128,819,724	128,819,844	TCTTCCAGATATCCTCGCTG	TATGACCTCGACTACGACTCG
MycCD	8q24	128,819,819	128,820,095	CTGGGATCTTCTCAGCCTAT	ACTCCTCTCACCATGAAGGT
SHGC-144185	8q24	128,928,822	128,929,092	TTATGGCCTTTTGGCATTCTCTA	TTGTGCACACAGCACTTCCTTAT
D8S1720	8q24	129,018,935	129,019,066	GTGCCACCTGCCTGAA	CCACTACCTATTTAGAGAGGCCA
SHGC-82342	8q24	129,133,364	129,133,574	CAGCAGTAAACTTGCCCTCAGA	TTAGGATTGGTTACTGGTGCTTT
STS-T95511	8q24	129,182,434	129,182,671	AGAATTTAATAGACACGAGGCCG	CTCCACCTGGACCTTATGGC
SHGC-84534	8q24	129,586,298	129,586,601	GGATTTTGGAAGGTAATGGGAAG	CCATTCCCTTGTCCACTCTTTTA
SHGC-142498	8q24	130,124,166	130,124,446	ATTGCTGCATTGTGCTAAGGAAC	CTGGGTCACTGTGTTTTCTCTCA
D8S1535	8q24	130,163,345	130,163,600	CAATTCCTTTGAATATTGCTTGC	TTGAAAAGTGCTAAGGAAACTGC
RH66480	8q24	130,297,999	130,298,125	CCACTTAGGGTCTGCCACATTGG	CTGGCAGAATGAATCACTTGG
MGC27434_KL	8q24	130,433,985	130,434,150	TGCAGGTTCACTGGAAATGA	AGGAGCGAAGAGTGTGGAGA
D8S1207	8q24	130,435,544	130,435,670	CTGTTGTGGGGGTGAACTGG	TGTGTCCTGGAAGGTGGTGG
STS-AA009754	8q24	130,451,661	130,451,862	CTGACTCAAAAAGTTGGAGAAACC	ATTTGCTTAATTCTGGAGCATAGC
SHGC-144692	8q24	130,559,142	130,559,341	AAACCAGGTCAGATGACTCACAT	GAGTTAAGACTATGGGCTTTAGGGC
MGC27434_IJg	8q24	130,562,018	130,562,171	CCCAGGGAAAGAGAGAGGAA	GAGCAGCCTTTTCAGGATTG
SHGC-151257	8q24	130,691,376	130,691,566	GGGTGTTAACATGAAAAGGAAACTG	CATGCATAGGTCTCAATTTCTGG
LOC389687_AB	8q24	130,709,636	130,709,803	TAGTGAGGCCCCAAATCAAA	AGCAGGGTCTGGACATGAAG
D8S1780	8q24	130,745,141	130,745,289	TACACTCCAGCCTGGG	AATTTCTGATTATTAGGAACACAC
MGC27434_GH	8q24	130,761,576	130,761,666	CTTGGTGTCTGGGAGGTCAT	AAACTTGGCTGCTGAATTGG
D8S1732	8q24	130,808,370	130,808,491	GTGGGATACCCCCATGT	ACTTCAGGGGCCATGTG
WI-15117	8q24	130,921,071	130,921,177	ATTCTGTTCCAGCCCCAAAG	GCCTCCTTCCTAAGCCTCAT
D8S1968	8q24	130,922,941	130,923,229	TTCTTTAGACATGAACATGCATACG	GCATGGCAAAACTGCCTATT
D14S609	14q11	19,620,035	19,620,205	AGCTCTAACAGTTTTGTCATGG	TATGTGCCCTAAAGGAAGCA
WI-18343	14q11	19,827,156	19,827,305	ATTCTGTTCCAGCCCCAAAG	GCCTCCTTCCTAAGCCTCAT
A002Q36	14q11	19,849,443	19,849,633	GCATGATCTTAGATCACTAAAG	CAGCTTTTTTAGTTTTGTCTC
G36285	14q11	19,888,995	19,889,104	CTTGGCCTTGTTGAGATTGC	CTGGCCTGTTACTCTTAGGG
G35564	14q11	19,932,346	19,932,445	GTGTACTGACCAATGTAGCC	GACTGCCTTCAAAACCTAGC
GDB:451907	14q11	19,993,759	19,994,089	TCCCTTGATGTACGGTAAG	TCTTCTTAATCCAGGCTCG
SHGC-36712	14q11	19,995,900	19,995,978	TAACCTCCCCCAAAGACTGA	TCCTCCCGACCTTTTTTACC
humpnu2	14q11	20,010,348	20,010,524	TGTCTCATACTAAGCACCGACC	TTCCAAGAACATCCCTCAGTCC
RH93852	14q11	20,121,904	20,122,071	CATCAGAGAATGACCTGTCAGC	CCCAGCTGCAAGTTTGTACA
D14S548	14q11	21,090,523	21,090,903	GGGCAACAGAGTGAGACTG	CCCCTTAGGAATTTTCCCAA
RH93874	14q11	21,609,607	21,609,734	CTGAATGTGCTTGAGACACACA	TATGCAGGACTCCAAAAGGG
D14S920	14q11	21,962,305	21,962,436	AAGCTAGTGTTTCTACCTTTCTTTC	ATGCTACAGTTCATTCCTTTGGCTC
GDB:593795	14q11	22,103,654	22,103,844	GGAGATGGCAGCTTATTGGA	GGCATGGAGTTCTTTAATTTGG
G35927	14q11	22,267,405	22,267,581	GGCAATCCACCCACCTTGG	CACCCACCAGAAATGTCAGG
A002Q41	14q11	22,459,580	22,459,794	CAGTAAAGGGCTATAACTTTATT	ATAATTAATTGCAGGGCT
D14S990	14q11	22,656,227	22,656,373	GTCCACTTGGTCATGGAAAC	AAGTTGCACTGTGACTGGG
RH75103	14q11	22,850,571	22,850,799	TCTCATTAAACTTCTGGGCTTTATT	TCCCCACTTATTCTAGGGCA
RH92419	14q11	22,918,608	22,918,752	CAATAAGTTCCCCTCCTCTGG	ATTCACTAGCCCCCAGCC
RH75843	14q11	23,677,858	23,677,990	TCTATTGAGAGCCCTCTCTCCC	ATTGTGCCTGAGAGGCAAGG
RH91413	14q11	23,846,989	23,847,157	ACACATACAAGCTCCAATGGC	AACCCTGGGATCTCAGCC
D14S1256	14q11	24,170,015	24,170,095	CCCGCTGTAATGAAACACCT	AGAGGTATTTATTCAGTTGCTGGC
D14S1163	14q11	25,068,521	25,068,681	AGGGGCTGGAGATTGATTG	GAGACAAGTGGGATAGGAGGG