Journal of Cellular Biochemistry 32:23–34 (1986)
Cellular and Molecular Biology of Tumors and
Potential Clinical Applications 47–58

Amplification of RNA and DNA Specific for erb B in Unbalanced 1;7 Chromosomal Translocation Associated With Myelodysplastic Syndrome

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Previous work has established the presence of an unbalanced chromosome abnormality [+der(1),t(1;7)(p11;p11)] in some therapy-associated myelodysplastic disorders. Recently the EGF receptor has been found to reside at 7p11. Using a probe specific for erb B oncogene, which encodes a truncated form of the EGF receptor, we examined RNA and DNA derived from bone marrow and peripheral blood mononuclear cells from three patients with myelodysplastic syndromes (MDS) and one with acute lymphocytic leukemia (ALL), all bearing an abnormal clone in their bone marrow with a similar unbalanced 1;7 translocation. DNA-excess slot blot hybridization to 5′-32p-labeled cellular RNA revealed from ten- to thirtyfold enhancement in accumulation of mRNA specific for erb B in both peripheral blood and bone marrow cells of the three MDS patients when compared to normal controls. In addition, enhancement of H-ras mRNA accumulation was detected in some, though expression of other genes such as actin, N-ras, myc, src, B-lym, and 20 other genes was not found to be enhanced. Increased erb B expression was not apparent in mononuclear cells from patients with other hematologic disorders such as chronic lymphocytic leukemia, Hodgkin's disease, or lymphoma. Southern blot analysis of restriction-enzyme-cleaved DNA from three MDS patients with an unbalanced 1;7 translocation revealed that erb B gene was amplified at least twentyfold in peripheral blood white blood cells, while levels of actin hybridization were comparable to those of the controls. No such amplification was evident in the ALL patient. Our data suggest that +der(1),t(1;7)(p11;p11) chromosomal anomalies can be specifically associated with amplification of erb B DNA and RNA sequences.

Key words: EGF receptor, oncogene, gene amplification

Chromosomal translocations involving +der(1),t(1;7)(p11;p11) have been found to be associated with myeloproliferative or myelodysplastic syndromes [1–3]. Frequently unbalanced 1;7 translocations of this type are correlated with a history of

Received February 21, 1986; revised and accepted June 3, 1986.

exposure to toxic substances such as alkylating agents, folic acid, environmental agents, or irradiation [1,4,5]. It is of interest that this unbalanced 1;7 translocation involves break and fusion points in chromosomal bands 1p11 and 7p11, and the loss of the 7q arm, centromere, and a small part of 7p arm adjacent to the centromere.

Recent studies have mapped the epidermal growth factor (EGF) receptor gene to 7p11 [6]. Sequence analyses have shown that the erb B oncogene of avian erythroblastosis virus encodes a truncated form of the EGF receptor that is in part responsible for the transforming potential of the virus. The cellular erb B protein product possesses a tyrosine-specific protein kinase activity and is capable of auto- and transphosphorylation [7]. This gene has been shown to be rearranged and/or amplified in a human breast cancer cell line [8], an epidermoid carcinoma cell line [9–10], and human brain tumors of gliomal origin [11]. One recent study, however, demonstrated an enhanced erb B mRNA expression but normal gene copy number in two pancreatic tumor cell lines displaying clonal structural alterations that resulted in overrepresentation of the short arm of chromosome 7 [12]. The similarities between the chromosomal abnormalities reported in pancreatic tumor cell lines and those cells used in this study are unknown.

The experiments described in this report were designed to investigate potential alterations in c-erb B gene structure and expression in cells derived from four patients with hematologic disorders and an abnormal clone containing +der(1),t(1;7)(p11;p11) in their bone marrow. We found amplification of the c-erb B gene as well as enhanced accumulation of erb B-specific mRNA in bone marrow and/or peripheral blood cells of three of these patients.

METHODS

cDNA Probes

pAM91 actin-specific probe was obtained from Dr. A. Minty [13]. c-H-ras, c-K-ras, c-mos, v-src, v-erb A, v-erb B, c-myc, v-fms, N-ras, v-raf, N-myc, and v-fos probes were obtained from American Type Culture Collection. B-lym probe was provided by Dr. G. Cooper, α-tubulin by Dr. C. Veneziale, transferrin receptor by Dr. F. Ruddle, IL2 (interleukin-2) and β-interferon by Dr. Taniguchi, v-fps and v-abl by Dr. A. Balmain, v-rel by Dr. H. Temin, v-bas and v-myb by Dr. R. Scott at the Mayo Clinic, v-fes by Dr. C.J. Sherr, p53 by Dr. Levine, v-sis by Dr. R. Gallo, α-interferon by Dr. C. Weissmann, and ornithine decarboxylase by Dr. Coffino.

Cytogenetics

Chromosome studies were done by using a direct technique for processing of bone marrow aspirates. Slide preparations were stained by GTG-banding or QFQ-banding or both [14]. In each case, 10–40 metaphases were examined.

Southern Blots

Bone marrow or peripheral blood buffy coat cells were obtained from four patients with a +der(1),t(1;7)(p11;p11) in association with hematologic disease (Table I). As a positive control for erb B amplification, the A431 cell line, known to have increased copies of erb B DNA [7], was used. DNA was purified from these cells by extraction in Tris-buffered phenol, precipitation from ethanol, and digestion with 10 μg/ml pancreatic ribonuclease followed by digestion with 50 μg/ml proteinase K [15].

TABLE I. Cytogenetic Data of Patients With an Unbalanced 1;7 Translocation*

Patient	Initial diagnosis	Exposure	Subsequent diagnosis	Specimen	Study	Fraction of metaphases with t(1;7) (karyotype)
1	Primary systemic amyloidosis	Melphalan Prednisone	Primary systemic amyloidosis and MDS	BM	1 2	27=46,XY/5=46,XY,−7,+der(1),t(1;7)(p11;p11) 24=46,XY/1=46,XY,−7,+der(1),t(1;7)(p11;p11)
2	RARS[a]	Phenylbutazone Anti-inflammatory agents Pyridoxine Folic acid	MDS	BM	1 2	8=46,XY/8=47,XY,+8/3=46,XY,−7,+der(1),t(1;7)(p11;p11) 17=46,XY/5=46,XY,del(7)(q22q34)/18=47,XY,+8
3	Multiple myeloma	Melphalan Prednisone	MDS	PB BM	1 2 3	20=46,XX 10=46,XX/10=46,XX,−7,+der(1),t(1;7)(p11;p11) 8=46,XX/12=46,XX,−7,+der(1),t(1;7)(p11;p11)
4	ALL[a]	Vincristine Methotrexate Adriamycin Cytoxan L-asparaginase	ALL remission		1 2	17=46,XY,t(9;22)(q34;q11) 3=46,XY/2=46,XY,t(9;22)(q34;q11)/ 5=46,XY,t(9;22)(q34;q11),+der(1),t(1;7)(p11;p11)

*ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; RARS, refractory anemia with ringed sideroblasts; BM, bone marrow; PB, peripheral blood.

[a]Fragile site studies on 50 QFQ-banded metaphases revealed the absence of fragile sites.

DNA was digested with the indicated restriction enzymes and subjected to 1.2% agarose gel electrophoresis in E buffer (50 mM boric acid-5 mM $Na_2B_4O_7 \cdot 10H_2O$-10 mM Na_2SO_4-0.1 mM Na_2 EDTA, pH 8.2). In all gels, standard molecular weight markers (Hind III digest of phage λ) were run, and DNA was visualized by staining in ethidium bromide.

DNA was denatured in the gel by incubating for 1 hr at room temperature in 0.5 M NaOH-0.6 M NaCl followed by 1 hr in 1.0 M Tris-HCl (pH 7.4)-1.5 M NaCl at room temperature. DNA from the gel was then transferred to nitrocellulose paper in 20 × SSC (300 mM Na citrate, pH 7.4, 3 M NaCl) for 18–48 hr at 4°C. Blot-bound DNA was hybridized to the appropriate heat-denatured nick-translated DNA probe in Southern hybridization buffer (2.5 μg/ml Poly A, 50 μg/ml herring sperm DNA, 0.1% SDS, 0.2% ficoll, 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 3 × SSC) at 65°C for 18 hr. The blot was then washed three times at 65°C for 1 hr each in 0.1 × SSC [16,17]. Blots were autoradiographed at −70°C to detect erb B-specific sequences.

Slot-Blot Hybridizations

RNA derived from bone marrow or peripheral blood buffy coat cells from each patient was purified in three steps: (1) alkaline phenol extraction, (2) precipitation from 3 M sodium acetate and (3) digestion with RNase-free DNase (Promega Biotec) [18].

Standard slot-blot hybridizations were used to obtain relative estimates of the amounts of specific RNAs within each RNA preparation. The technique of dotting excess clone-specific DNA and hybridizing to ^{32}P-labeled RNA has been used extensively as a means of measuring relative quantities of specific RNAs in a single preparation [19]. In all experiments, 1 μg double-stranded DNA in 1 M ammonium acetate was determined to be DNA excess for the reaction and was used in all experiments reported here. Filters were washed in 4 × SSC and baked in a vacuum oven at 80°C overnight. Prior to hybridization, filters were soaked in 1× Denhardt's solution (0.2% ficoll, 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidine), 3 × SSC (45 mM Na citrate, pH 7.4, 0.45 M NaCl) at room temperature for 1 hr followed by washing for 1 hr at 50°C in hybridization buffer (50% formamide, 1× Denhardt's solution, 10 μg/ml Poly A, 50 μg/ml herring sperm DNA, 3 × SSC).

Poly A^+ RNA was 5′-end labeled with ^{32}P as follows: 2 μg RNA were partially hydrolyzed with NaOH. After neutralization of the NaOH with HCl, the RNA was incubated at 37°C for 45 min with T4 polynucleotide kinase and 50 μCi γ-^{32}P-labeled ATP (3,000 Ci/mmol). RNA was separated from unincorporated ATP by Sephadex G50 column chromatography at room temperature in 3 × SSC. Prior to hybridization, RNA aggregates were broken up by heat-shocking the sample for 1 min at 90°C. The RNA was hybridized to the nitrocellulose filters dotted with DNA probes at 50°C for 18 hr. Filters were washed three times for 1 hr each in 3 × SSC at 65°C and three times for 1 hr each in 0.1 × SSC at 65°C. Nitrocellulose filters were set up for autoradiography with X-ray film at −70°C for 24 hr. After autoradiographs were obtained, microdensitometric analysis was performed.

RESULTS

Cytogenetic Studies

Table I summarizes the clinical and cytogenetic data for the four individuals in this study. Each patient had a history of drug therapy prior to the appearance of the

clone with an unbalanced 1;7 translocation. Patients 1 and 3 each had an abnormal clone where the only abnormality was a +der(1),t(1;7)(p11;p11). In patient 2 there were at least three different abnormal clones observed; one was trisomy 8, another had a deletion of part of the long arm of a chromosome 7, and the third clone had an unbalanced 1;7 translocation. Patient 4 was studied on two occasions. The first time the only abnormal cells observed were those with a Ph-chromosome. In the second study three of the cells had an apparent unbalanced 1;7 translocation in addition to a Ph-chromosome. Thus, the 1;7 translocation appeared in a subclone of a Ph-chromosome-positive clone. Figure 1 depicts representative karyotypes from bone marrow cells of two of the patients. The fact that these 1;7 translocations are present in only a percentage of the patients' cells and that some patients acquired the t(1;7) translocation after therapy demonstrates that the translocations are somatic, not constitutional.

DNA Studies

Southern blots were performed in order to determine whether t(1;7)(p11;p11) translocations involve amplification of the c-erb B gene. Figure 2 demonstrates amplifications of erb B-specific sequences in peripheral blood lymphocytes from three of four patients bearing a 1;7 translocation. (Similar results were obtained by using bone-marrow-derived DNA from patient 1; data not shown.) Densitometric analyses of these blots revealed levels of erb B-hybridizing sequences enhanced from 20- to 30-fold in three MDS patients compared to placental DNA. This is especially interesting in light of the absence of the 7q arm, the 7 centromere, and a bit of the 7p arm that accompanies the unbalanced 1;7 translocation. One would expect the loss of the chromosome 7p arm to result in decreased copies of c-erb B in the genome if this gene resided on the region of the 7p arm lost in this unbalanced translocation. As depicted in Figure 2, however, this is not the case in at least three of four individuals studied. It should be noted that DNA samples were not derived from cell lines but instead from bone marrow white blood cells taken directly from the patient. (Attempts to establish clonal lines from these individuals were unsuccessful.) Therefore, the cells are a mixture of normal cells and those with a 1;7 translocation. While amplification of c-erb B sequences is evident, it may actually be much higher than observed if experiments could be done with purified cell populations with a 1;7 translocation. Restriction enzyme patterns of erb B sequences from peripheral blood cells of patients with a 1;7 translocation suggest that in some individuals there is selective amplification of specific bands while in others the entire gene is amplified equally. No unusual bands were evident, which implies that amplification occurs over a span of DNA larger than the c-erb B gene itself.

To ensure that equal amounts of DNA were loaded per lane, gels were stained with ethidium bromide prior to transfer. In all cases, relatively equal amounts of DNA were apparent in each lane. In addition, blots were dehybridized overnight in H_2O at 65°C and then rehybridized to cDNA probe specific for actin (data not shown). These results established that each lane contained relatively equal amounts of actin-hybridizing DNA.

RNA Studies

Experiments were performed to determine whether the erb B amplification evident in Figure 2 was accompanied by a concomitant enhancement in erb B-specific mRNA accumulation. Total cellular RNA derived from bone marrow and/or periph-

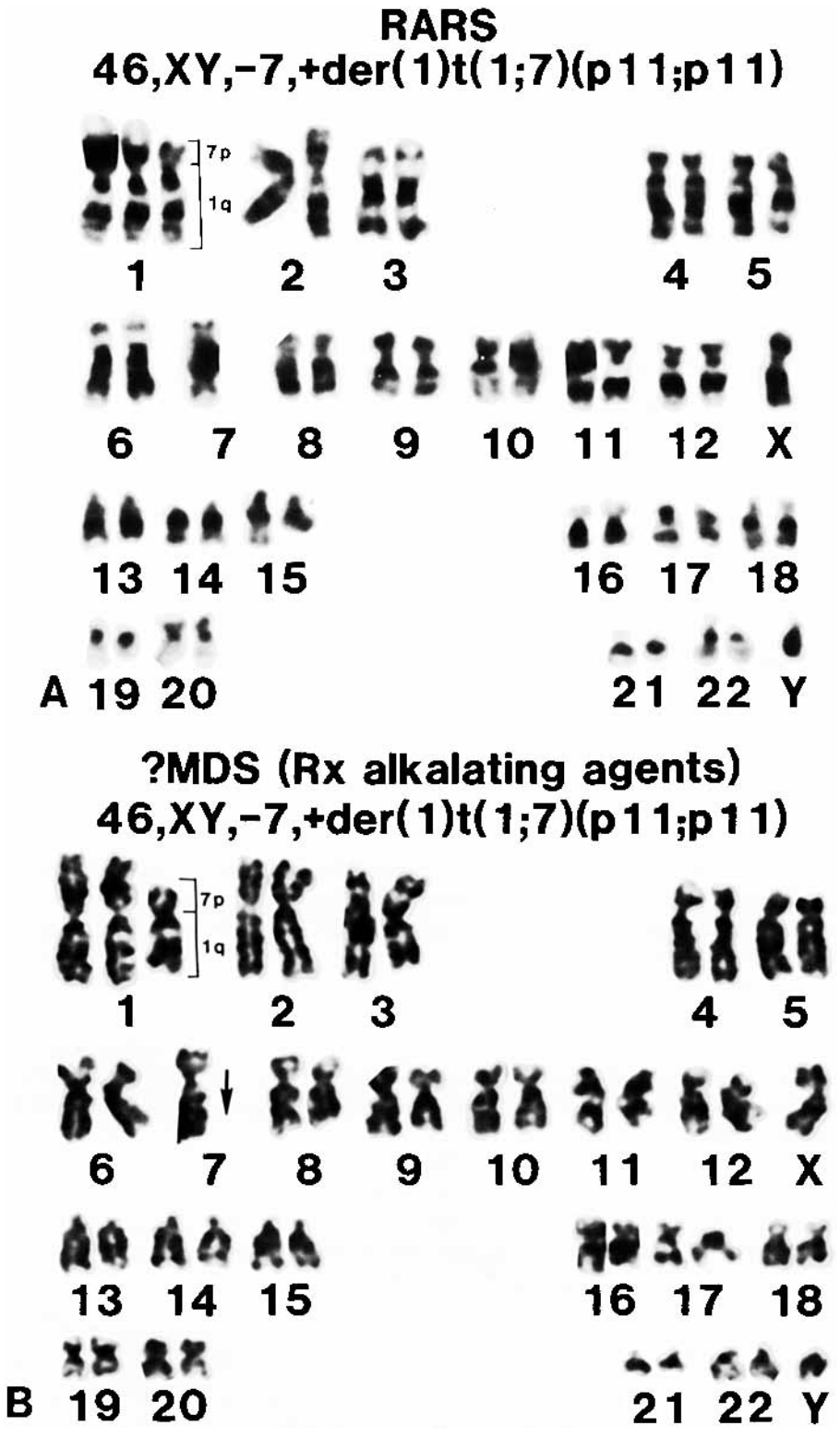

Fig. 1. Representative karyotypes derived from bone marrow cells of four patients with an abnormal clone containing +der(1),t(1;7)(p11;p11). A) Patient 2. B) Patient 3.

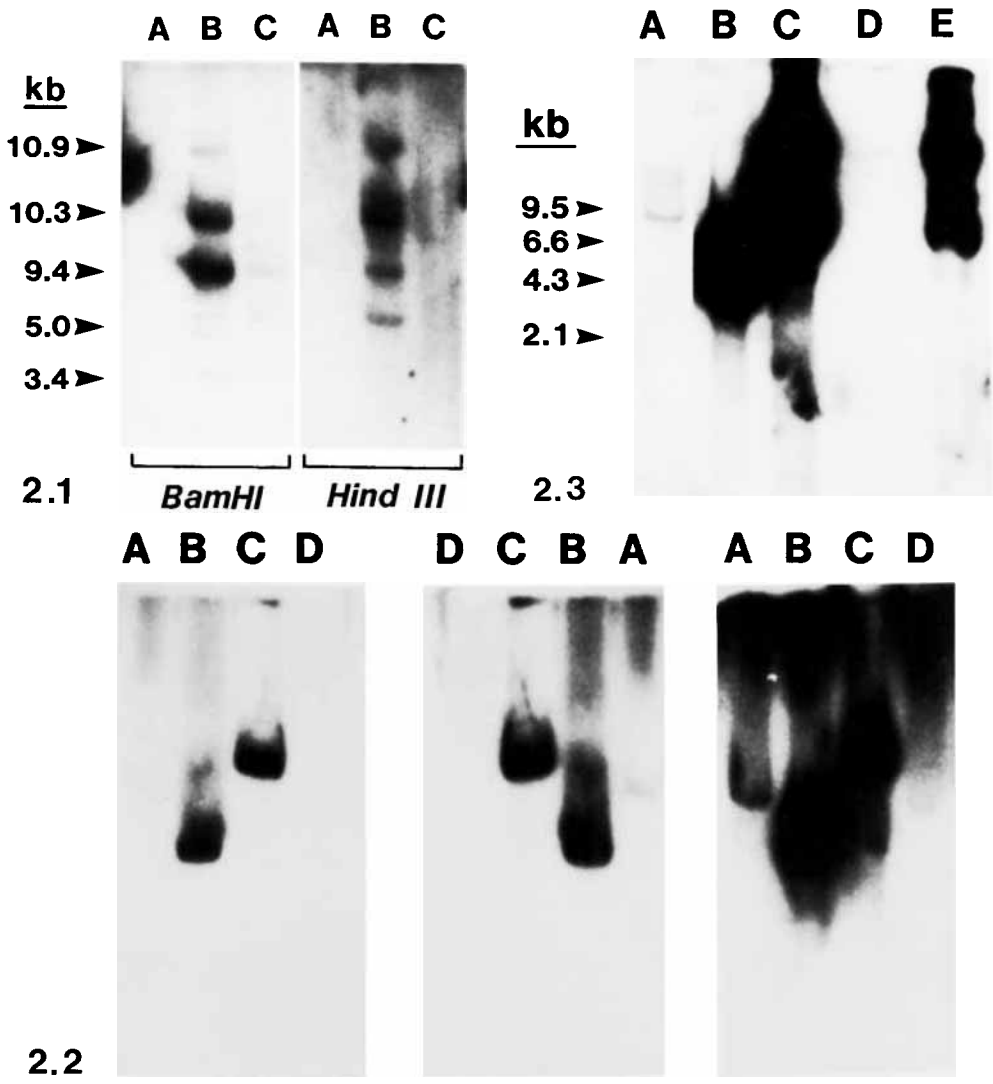

Fig. 2. Southern blot analyses of DNA derived from cells of patients bearing t(1;7)(p11;p11) hybridized to nick-translated v-erb B DNA. 2.1) DNA from (A) control cells, (B) patient 1, and (C) placenta was digested with Bam HI or Hind III prior to electrophoresis, blotting, and hybridization. 2.2) Bam HI-digested DNA derived from (A) A431 cells, (B) patient 1, (C) patient 2, and (D) placenta hybridized to v-erb B. The left panel represents 12-hr exposure, the middle panel depicts a 24-hr exposure, and the right panel a 48-hr exposure. 2.3) Eco RI digest of DNA derived from (A) patient 4, (B) patient 3, (C) patient 2, (D) placenta, and (E) A431 cells.

eral blood lymphocytes was 5′end-labeled with ^{32}P in a kinase reaction and hybridized to an excess of clone-specific DNA immobilized onto nitrocellulose paper. These dot-blot hybridizations can be used to obtain a relative estimate of the quantity of clone-specific RNA present in the preparation. Figure 3 depicts the results of a representative dot-blot hybridization. The autoradiographs of such blots using equivalent counts and exposure times for each were analyzed by microdensitometry. The results representing three independent observations are listed in Table II. From these it is clear

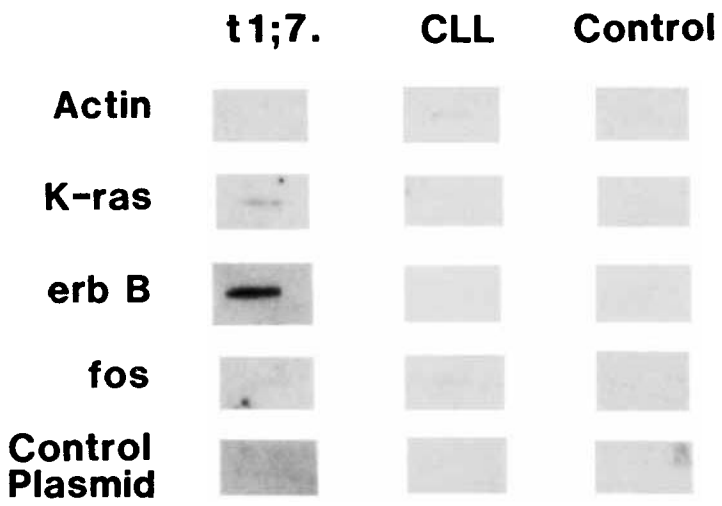

Fig. 3. Autoradiograph of slot blot hybridization of clone-specific DNA to 5′-labeled RNA derived from peripheral blood lymphocytes of t(1;7)(patient 1), control (PHA-activated peripheral blood white blood cells derived from a normal control), and CLL (peripheral blood white blood cells from T-cell chronic lymphocytic leukemia).

that the peripheral blood lymphocytes from three patients with amplified c-erb B DNA (those with MDS) also exhibited increased accumulation of mRNA specific for c-erb B. RNA derived from peripheral blood cells of the Ph-chromosome-positive ALL patient in which no c-erb B amplification was evident also demonstrated no such enhancement in erb B expression. For patients 1 and 4, RNA derived from bone marrow and peripheral blood lymphocytes both demonstrated enhanced erb B mRNA accumulation (data not shown).

Interestingly, studies of other gene sequences also revealed enhanced expression of K-ras, fos, and fes variably in some patients but not others. The significance of this is unclear, though similar enhanced expression of non-amplified c-oncogenes has been detected in many different tumor systems [20,21]. In addition, little change in gene expression was observed in genes associated with chromosome 1, such as B-lym, src, and N-ras.

DISCUSSION

Unbalanced translocations between chromosomes 1 and 7 associated with loss of a small part of a chromosome 7p arm, chromosome 7 centromere, and 7q arm have been correlated with myelodysplastic and myeloproliferative disease; the breakpoints are usually at 1p11 and 7p11. Frequently this chromosomal abnormality is linked with a history of exposure to toxic therapeutic or environmental agents [1–4]. In this study we examined cells from four patients with an abnormal clone in their bone marrow containing an unbalanced t(1;7)(p11;p11). Three of these patients had a history of exposure to cytotoxic drugs (especially alkylating agents) prior to the detection of the translocation. One patient had received a variety of anti-inflammatory agents including phenylbutazone.

TABLE II. RNA Dot Blot Hybridization*

	Control[a]	PBL 1[b]	PBL 2[b]	PBL 3[b]	PBL 4[b]
Actin	1.0	1.1	1.3	1.0	1.1
Transferrin receptor	HND[c]	3.1	HND	HND	10.4
α-tubulin	HND[d]	HND	HND	HND	HND
H-ras	HND	4.7	HND	HND	12.1
K-ras	HND	HND	HND	HND	HND
α-interferon	HND	HND	HND	NT[e]	NT
β-interferon	HND	2.8	HND	NT	NT
B-lym	2.0	1.7	HND	5.1	HND
fes	HND	HND	HND	HND	HND
fps	HND	HND	HND	HND	1.2
pBR322[f]	HND	HND	HND	HND	HND
sis	HND	HND	HND	NT	NT
erb B	HND	14.4	33.7	10.4	HND
myc	HND	HND	HND	HND	HND
mos	HND	3.8	HND	2.1	12.7
myb	HND	HND	21.4	4.3	20.6

*Analysis by microdensitometry. All data reported in arbitary units. All units determined by 1-wk exposure of 3.7×10^5 cpm hybridized per blot. All data reflect mRNA accumulation relative to the amount of actin-mRNA in control cells.

[a]PHA-activated PBL from healthy donors; RNA from tonsillar tissue gave similar results.

[b]Cells from patient bearing an abnormal clone with unbalanced 1;7 translocation.

[c]Hybridization not detected.

[d]Also negative for all cell types: ornithine decarboxylase, p53, abl, raf, fms, N-myc, bas, rel, erb A, N-ras, src.

[e]NT, not tested.

[f]Negative control; parent plasmid.

Since c-erb B gene (EGF receptor gene) has recently been mapped to 7p-11, we examined DNA and RNA derived from cells presumably bearing the chromosomal abnormality. These experiments revealed 20- to 30-fold amplification of c-erb B-specific DNA in three of the four patients. In addition, we found evidence for enhanced erb B mRNA expression in the three patients exhibiting amplified DNA.

Interestingly, the cells from the three patients with a 1;7 translocation and myelodysplastic syndrome displayed amplified c-erb B DNA while cells from the ALL patient with a similar 1;7 translocation showed no such DNA increase. It is possible that c-erb B amplification was not detected in this individual because (1) intensive chemotherapy suppressed or killed cells bearing the 1;7 translocation or (2) cells bearing the 1;7 translocation were present at too low a cell density to detect the amplification. On the other hand, the 1;7 translocation evident in the ALL patient may have involved translocation without amplification of c-erb B sequences.

Our attempts to obtain cell lines from these individuals were unsuccessful. Therefore, all our studies utilized heterogeneous cell samples with less than 50% (as determined by cytogenetics) of cells bearing a 1;7 translocation (see Table I). It is likely that all estimates in this report of levels of erb B gene amplification and mRNA accumulation present in these cells are actually low and reflective of the amount per total cell population rather than per cell bearing a 1;7 translocation.

The 1;7 translocation is accompanied by a loss of a part of chromosome 7p arm and all of the chromosome 7 centromere and 7q arm. Therefore, one may postulate two models for the location of c-erb B gene following a 1;7 translocation: (1) c-erb B

gene may be lost with a small part of the 7p arm in the unbalanced chromosomal abnormality or (2) c-erb B gene may be translocated with most of 7p to chromosome 1p11. If the former were true, one would predict the amount of c-erb B-specific DNA to be decreased based on gene dosage [22]. However, since we have detected amplification of c-erb B gene sequences, we favor the latter model associating the translocation with c-erb B gene amplification. In addition, cytogenetic analysis did not reveal the presence of double minutes or homogeneous staining regions to explain this amplification.

7p11 is the site not only for the location of the c-erb B gene but also for a folic acid-sensitive fragile site found in some individuals [23]. Experiments were performed to determine whether these patients also had a fragile site at 7p11 which might affect a predisposition toward a 7p11 breakpoint in response to therapy. Of the two patients tested, fragile sites were not detected in peripheral blood cells cultured in the presence of 5-fluorodeoxyuridine (FUdR; see Table I). This suggests either that the methods needed to detect fragile sites are not sufficiently sensitive or that fragile sites are not necessarily correlated with the origin of such 1;7 translocations.

Recent results by King et al [24] have identified several human tumor cell lines which all display increased c-erb B mRNA expression by three potential mechanisms: (1) gene amplification with rearrangement and altered transcript size, (2) gene amplification without rearrangement and normal mRNA size, and (3) enhanced mRNA expression in the absence of gene amplification. The results presented in this report suggest that the amplified c-erb B gene is associated with 1;7 chromosomal translocation. While the presence of a translocation must involve a genomic rearrangement it is not clear that the c-erb B gene itself is rearranged in all patients. Southern blots (Fig. 2) demonstrated that the 1;7 translocations of patient 1 and patient 3 involved amplification of c-erb B in the absence of erb B rearrangement. This suggests that the amplification event itself involved DNA fragments larger than the c-erb B gene and that the translocation-specific breakpoint on chromosome 7 is located centromeric to c-erb B. On the other hand, Southern blot analyses of DNA derived from patient 2 (Fig. 2.2) demonstrate amplification accompanied by rearrangement within c-erb B gene. Clearly both of these mechanisms can be involved in 1;7 translocations associated with myelodysplastic syndromes.

Many have suggested that drugs that block DNA synthesis cause gene amplification by allowing for local areas of overreplication [25]. The fact that these patients developed a 1;7 translocation associated with c-erb B amplification suggests a potential role for specific therapeutic agents (see Table I) in c-erb B amplification. Moreover, most oncogene amplifications have been associated with aggressive tumors, occurring as late-stage events in oncogenesis [26]. In these patients, MDS represents a preleukemic syndrome with low levels of cellular proliferation. Their subsequent diagnoses of MDS differed from their initially diagnosed diseases. This suggests that gene amplification may also represent an early initial event that later leads to cellular transformation.

It has been hypothesized that specific chromosomal abnormalities highlight areas of active gene transcription in differentiated cells [27]. The data presented in this report suggest that the region of chromosome 7 involved in the translocation encompasses the c-erb B gene. This gene is not normally expressed at a detectable level in bone marrow or peripheral blood cells (Woloschak, unpublished observations). Clearly it is possible that other genes located on chromosome 7 are also

involved in the translocation, especially in light of the apparently large size of the amplified DNA. Recent work by Mark et al [28] has isolated another oncogene at 7p11.1 that may also be associated with this and other chromosome 7 abnormalities. The identities of the gene sequences on chromosome 1 that are involved in this translocation are unknown. RNA studies of chromosome 1-associated genes such as src, B-lym, and N-ras revealed little enhancement of expression in patients bearing an abnormal clone with a 1;7 translocation compared to controls (see Table II). Future cloning experiments may help to characterize these genes.

ACKNOWLEDGMENTS

These studies were supported by ACS IM-348, Fraternal Order of Eagles grant 50, and the Mayo Foundation. The authors wish to thank Kathy Jensen for typing the manuscript and Mary Jane Doerge for technical assistance.

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