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18 April 1990; accepted 16 July 1990

Cell-Adhesive Motif in Region II of Malarial Circumsporozoite Protein

KATHRYN A. RICH,* F. W. GEORGE IV, JUDY L. LAW, W. JOHN MARTIN

The segment of the malarial circumsporozoite (CS) protein designated Region II is highly conserved among different malarial species. A similar sequence is also present in several other proteins, including thrombospondin, properdin, and a blood-stage antigen of *Plasmodium falciparum*. By means of peptides synthesized from sequences of the *Plasmodium vivax* CS protein in the vicinity of Region II, it was found that two overlapping 18– to 20–amino acid peptides promoted the adhesion of a variety of human hematopoietic cell lines. The amino acid sequence valine-threonine-cysteine-glycine (VTCG), contained within this common motif, was shown to be the critical sequence for the observed cell-adhesive properties.

THE MALARIAL CIRCUMSPOROZOITE (CS) protein found on the surface of mature sporozoites ranges from 40 to 60 kD in different species and varies in immunological reactivity (*1*). The central region of the CS protein consists of tandem repeats that show marked differences among species in their number, length, and amino acid sequence (*2*). However, there are two regions of conserved amino acid sequences (designated Regions I and II) (*3*), one of which is present on either side of the repeats (Fig. 1). Region II shows a particularly high degree of conservation in all species of malaria parasites sequenced to date (*4, 5*), suggesting that it may have an important biological function. Furthermore, it has recently been recognized that a sequence that is very similar to Region II of CS proteins is found in three apparently unrelated proteins: in properdin (*6*), which stabilizes the $C3b_nBb$ enzyme complex of the alternate complement pathway, in the type 1 repeats of thrombospondin (*7*), and in a blood-stage antigen of *Plasmodium falciparum* called thrombospondin-related anonymous protein (TRAP) (*8*). So far, no function has been ascribed to this conserved motif.

Using a series of overlapping, synthetic 15– to 20–amino acid peptides (designated PV-21 to PV-24; see Fig. 1) corresponding to sequences in the vicinity of Region II, we have recently defined an antigenic region within the CS protein of *P. vivax* that is recognized by T lymphocytes. The epitope is contained in peptide PV-23, which is immunogenic in mice and shows cross-reactivity with CS protein of *P. vivax* (*9*). We also tested the various peptides for their ability to bind peripheral blood lymphocytes. Initial studies with peptides (*10*) dot-blotted onto nitrocellulose showed adhesion of lymphoid cells to PV-22 and PV-23. Subsequently, a quantitative assay with ^{51}Cr-labeled cell lines and peptide-coated microtiter plates was developed (*1*). Peptides PV-22 and PV-23 promoted the attachment of a variety of human hematopoietic cell lines (Table 1). Several, but not all, T cell and myeloid cell lines showed high levels of adhesion (60 to 76% of added cells bound); whereas B cell lines, the monocytoid line U937, and small cell lung carcinomas adhered to a lesser degree. In contrast, attachment of all cell lines to PV-21, PV-24, or bovine serum albumin (BSA) averaged 4%. Cell attachment to peptides PV-22 and PV-23 was dependent on temperature, but not on the presence of Ca^{2+} or Mg^{2+} (*12*).

We also found that the peptides PV-22 and PV-23 were active in soluble form and capable of inhibiting cell attachment to peptide-coated plates. For these experiments we used the T cell line, CEM, and the myeloid cell line, K562. Cells were incubated for 1 hour at 37°C in the presence of peptides and added to plates coated with PV-22 or PV-23. Incubation with either PV-22 or PV-23 (100 μg/ml; ~0.05 mM) resulted in a 70 to 90% inhibition of subsequent cell attachment to PV-23–coated plates (Table 2, series A). Identical results were obtained with PV-22–coated plates; that is, incubation of the cells with either peptide was effective in preventing subsequent attachment. Additional experiments (*12*), in which the cys-

Table 1. Attachment of cells to microtiter plates coated with *P. vivax* synthetic peptides. Results are presented as the percentage of the total cells which attached to the plates calculated as [counts per minute (cpm) in well after washing]/(cpm added to well) × 100 = (percent attachment). The standard deviation of triplicate estimations ranged from 1 to 10% with an average of 2%. The degree of cell attachment to PV-21 was similar to that shown for PV-24. Results of a representative experiment are shown. SCLC, small-cell lung cancer.

Cell line	Cell type	Percentage of cells attached to peptide-coated plates			
		Control	PV-22	PV-23	PV-24
CEM	T cell	6	66	68	4
HSB-2	T cell	5	60	76	9
MOLT-3	T cell	1	9	11	2
K562	Myeloid	3	69	70	3
KG-1	Myeloid	2	60	64	3
HL-60	Myeloid	3	6	7	4
IM-9	B cell	3	13	18	2
Raji	B cell	13	22	28	16
RPMI 7666	B cell	3	12	15	3
U937	Monocytoid	6	12	6	5
H82	SCLC	3	16	14	2
H446	SCLC	3	8	13	3

Department of Pathology, University of Southern California School of Medicine, Los Angeles, CA 90033.

*To whom correspondence should be addressed.

teine residues in peptides PV-22 and PV-23 were reduced and alkylated, demonstrated that the oxidative state of the cysteine is not important in the cell adhesion phenomenon, since these modified peptides were capable of both promoting and inhibiting cell attachment in a manner similar to that of the original peptides. In contrast, incubation of cells with PV-21 (which also contains a cysteine residue) or PV-24 had no effect on attachment to PV-22– or PV-23–coated substrates.

To assess the possible relevance of these observations to the interaction of the malarial sporozoite with host cells, we tested several different recombinant CS protein preparations in the cell adhesion assay. The CS proteins vivax-1, vivax-2, and vivax-3 differ in the extent of their representation of the complete CS molecule (*13*) (Fig. 1). Vivax-1, which is currently being evaluated in clinical trials, represents amino acids 82 to 315 and thus contains only a portion of Region II, but none of the amino acids in PV-23. Vivax-2 (amino acids 82 to 340) contains Region II, all of the amino acids in PV-22 and PV-23, but only half of PV-24. Vivax-3 represents amino acids 28 to 340, but contains only two of the tandem repeats and thus is an abbreviated version of vivax-2. Incubation of cells with the CS proteins vivax-2 or vivax-3 (100 μg/ml) resulted in a 45 to 58% inhibition of cell attachment to PV-23–coated substrates (Table 2). The CS protein vivax-1, which lacks the putative cell adhesive region, was inactive (2 to 12% inhibition).

Since PV-22 and PV-23 share a region of 12 amino acids (Fig. 1), we proposed that this overlapping sequence may mediate the observed cell adhesion phenomenon. To test this hypothesis, a ten–amino acid peptide (designated PV-22/23) corresponding to the overlap region, but excluding amino acids in PV-24, was synthesized. A control

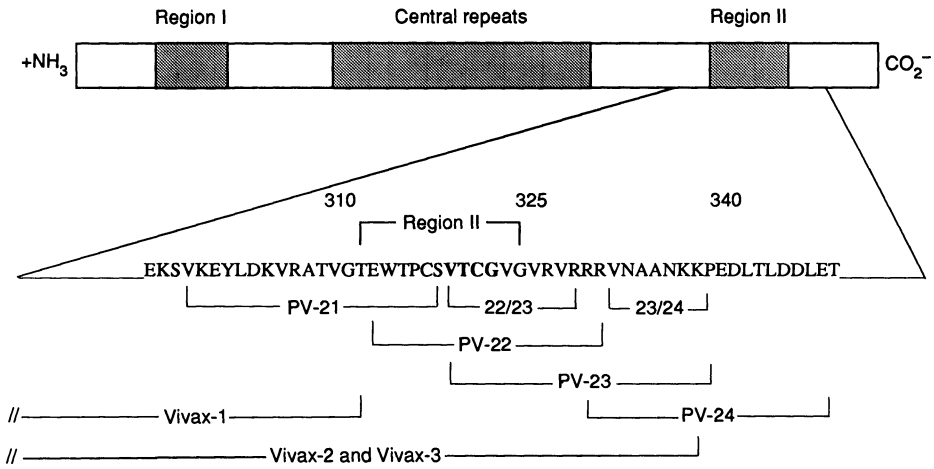

Fig. 1. Location of synthetic peptides of *P. vivax* CS protein. A schematic diagram representing the single-letter amino acid sequence (*14*) for the *P. vivax* CS protein, based on a publication by Arnot *et al.* (*28*) and corresponding to the sequences for the vivax CS protein preparations from Chiron Corporation (Emeryville, California). The sequences for peptides PV-21 and PV-23 were based on a previous publication by Arnot *et al.* (*4*) and hence contained the amino acids LET at position 305 to 307, and the amino acid S at 328, respectively. Regions I and II are nonpolymorphic regions common to all malaria species.

peptide of eight amino acids (PV-23/24) corresponding to the overlap of PV-23 and PV-24, was also synthesized. Incubation of CEM or K562 cells with PV-22/23 (1000 μg/ml) before their addition to PV-23–coated plates, resulted in a significant inhibition (59 to 72%) of cell attachment (Table 2, series B). However, incubation of cells with the overlap peptide PV-23/24 was without effect. The sequence Val-Thr-Cys-Gly-Val-Gly-Val-Arg-Val-Arg (VTCGVGVRVR) (*14*), which represents the overlap peptide PV-22/23, therefore contains a sequence that mediates cell adhesion. Comparison of CS protein with thrombospondin, TRAP protein, and properdin shows that the four–amino acid sequence VTCG is highly conserved in these proteins (*6*), and thus may represent the critical adhesive sequence. Two additional peptides were synthesized, one containing the sequence VTCG and the other containing the sequence VGVRVR, the latter being the remainder of the overlap peptide PV-22/23. The peptide VTCG shows a significant inhibitory activity (43 to 49%) on cell attachment, although in comparison with the peptides PV-22 and PV-23 the potency is somewhat lower (Table 2). The peptide VGVRVR was without effect. Similarly, incubation of cells with the previously identified adhesive peptides Arg-Gly-Asp-Ser (RGDS) and Tyr-Ile-Gly-Ser-Arg (YIGSR) resulted in less than 10% inhibition of cell attachment to PV-22– or PV-23–coated plates (*12*).

To further test this hypothesis, a shortened PV-23 peptide, which lacks the NH_2-terminal four amino acids VTCG (designated PV-23S) was synthesized. This peptide did not mediate cell attachment when coated on plastic microtiter plates (only 3% CEM

Table 2. Inhibition of cell attachment by *P. vivax* CS proteins and synthetic peptides. ^{51}Cr-labeled cells were incubated with CS proteins or peptides at 100 μg/ml (for series A) or peptides at 1000 μg/ml (for series B) for 1 hour at 37°C and added to PV-23–coated plates. Experiments were completed as described (*10*). Results are presented as percent inhibition of attachment to PV-23–coated plates (mean ± SE) calculated from two series (A and B) of four to seven different experiments. Total binding (incubation with BSA alone) ranged from 68 to 82%. Similar results to those obtained from PV-24 were observed after incubation with PV-21 (3 to 10% inhibition). Analysis of variance was used to determine the significance of treatment effects; group means were then compared to control (BSA alone) with Duncan's multiple range test (*$P < 0.01$). Cell viability (as assessed by trypan blue exclusion) remained unaltered (>95%) in the presence of peptides.

Experimental series		Percent inhibition after incubation with					
A	Cell line	PV-22	PV-23	PV-24	Vivax-1	Vivax-2	Vivax-3
	CEM	89.0 ± 3.8*	85.7 ± 2.2*	5.8 ± 2.6	12.3 ± 4.5	45.4 ± 13.5*	58.3 ± 7.2*
	K562	87.5 ± 1.6*	86.9 ± 4.0*	4.0 ± 2.1	1.8 ± 1.8	54.6 ± 10.1*	53.9 ± 9.2*
B	Cell line	PV-23	PV-23S	PV-22/23	PV-23/24	VTCG	VGVRVR
	CEM	88.0 ± 2.2*	2.2 ± 1.7	59.3 ± 8.7*	0.7 ± 0.7	43.0 ± 5.4*	2.3 ± 1.8
	K562	87.5 ± 3.1*	9.0 ± 4.7	71.8 ± 12.1*	2.0 ± 2.0	49.2 ± 7.8*	7.6 ± 3.4

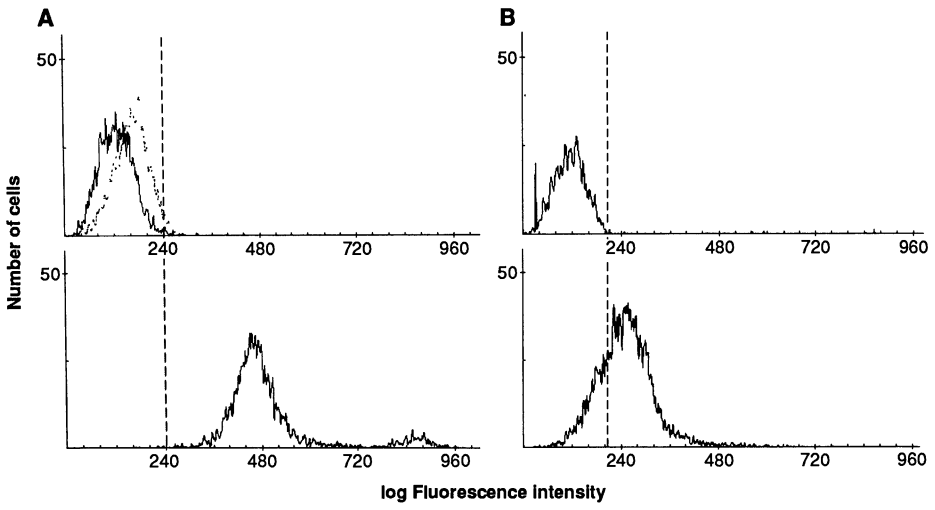

Fig. 2. Fluoresence staining of CEM cells incubated with FITC-labeled *P. vivax* synthetic peptides or CS proteins. (**A**) Staining of cells with either PV-23S (—) or PV-21 (. . .) (upper panel) or PV-23 (lower panel). (**B**) Staining of cells with either vivax-1 (upper panel) or vivax-3 (lower panel). The flow cytometer was gated with the use of unstained cells such that positive fluorescence gave signal intensities greater than 240 (A) or 210 (B) as indicated by the dashed line. The staining pattern for cells incubated with PV-23S, PV-21, or vivax-1 was essentially identical to that of unstained cells. When tested in the cell adhesion assay, the activity of PV-23 was not found to be altered by conjugation with FITC.

cells were bound) nor did it inhibit cell attachment to PV-23–coated plates (Table 2B). The conclusions from these studies were further confirmed by flow cytometry experiments with fluorescein-labeled peptides (*15*). Binding of fluorescein isothiocyanate (FITC)–labeled PV-23 was readily demonstrated on CEM cells (100% staining), whereas minimal staining occurred with either FITC-labeled PV-23S (1.4%) or PV-21 (7.7%) (Fig. 2A). Consistent results were obtained in a comparison of CEM cells stained with FITC-labeled vivax-3 (77% staining) and vivax-1 (0.6%) (Fig. 2B). We have also shown by flow cytometry that normal peripheral blood lymphocytes, polymorphonuclear cells, and monocytes exhibit a low level of binding to FITC-labeled PV-23 (*12*). The level of binding increases upon activation, suggesting an increase in the levels of adhesive receptors at the cell surface.

VTCG-mediated cell attachment is sensitive to trypsin, suggesting involvement of a protein component (*12*). Furthermore, the myeloid line K562, which we have shown by flow cytometry does not express CD4, CD8, or major histocompatibility complex class I antigens, is highly adherent in our assay, indicating that the adhesive receptor is probably not one of these molecules. In addition, it would appear that hematopoietic cells do not bind to this cell-adhesive motif via either fibronectin-type (*16*) or laminin-type (*17*) receptors since neither the tetrapeptide RGDS nor the pentapeptide YIGSR were found to be inhibitory in our assay. Several gene families are emerging in the field of cell-cell recognition and cell-matrix interactions for hematopoietic cells. Those include the integrin (*18*) and immunoglobulin supergene (*19*) families and a newly identified family of adhesion proteins [ELAM-1 (*20*) and lymph-node homing receptor (*21*)] involved in leukocyte-endothelial cell interactions. The continual discovery of new leukocyte adhesion receptors, such as the Hermes/CD44 antigen (*22*) and additional members of the very late antigen family (*23*), for which neither the ligands nor the functions have been clearly defined, suggests a wealth of novel leukocyte adhesion capabilities.

Here we demonstrate the presence of a cell-adhesive motif in Region II of malarial CS protein. The studies with the shortened PV-23 peptide demonstrate a critical role for the sequence VTCG in the cell-adhesive properties of PV-23. The lesser degree of inhibition of cell attachment observed with the small peptide VTCG, in comparison with PV-23 itself, may indicate some complementary role for the additional amino acids in the larger peptide. While the affinity of binding of VTCG for its receptor cannot be estimated from the present data, the concentration of peptide (1000 μg/ml or 2 mM) used in the inhibition studies is comparable to that used by others for RGD-containing peptides of similar size (*24*). Substitution studies with a panel of peptides will be required to determine the contribution of individual amino acids in the VTCG sequence. Furthermore, preliminary experiments with a new peptide, GVCT, in which the amino acids of VTCG have been rearranged, show absence of activity in the inhibition assay.

Although the oxidative state of the cysteine residue did not appear to influence the cell-adhesive properties of the peptides examined in this study, in larger proteins cysteine residues may be involved in either inter- or intramolecular disulfide linkages. Information is not presently available as to whether the cysteine residue in the VTCG sequences of CS, thrombospondin, and properdin is free or bridged to other cysteine residues in these proteins. From a detailed analysis of the number of residues between half-cysteines in globular proteins, Thornton (*25*) found that there is a strong preference for the shorter connections, the most frequent separation being 10 to 14 residues. Thus, half-cysteines that are close together in sequence preferentially form disulfide bridges. The CS protein contains two pairs of cysteines, at residues 316 and 320 (VTCG), and at residues 350 and 355 (*3*). These pairs of cysteines may form a single intramolecular loop; however, others have speculated that two small loops are formed (*26*), which would tend to expose the VTCG sequence.

It is of interest that the deduced amino acid sequence of a protein from the sexual stage of *P. falciparum* (Pfs 25) also contains the sequence VTCG. It was suggested that this protein may be involved in binding of the parasite to receptors on mosquito epithelial cells to facilitate invasion (*27*). Whether the conserved motif VTCG in such diverse molecules as thrombospondin, properdin, and malarial parasite proteins has any common function related to the cell-adhesive properties of this sequence is not known. An understanding of the cellular receptors for this adhesive sequence may shed light on the function of this motif.

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10. Peptides PV-21, PV-22, PV-23, and PV-24 were synthesized at the University of Southern California Norris Cancer Center Microchemical Core Facility on an Applied Biosystems model 430A automated peptide synthesizer with the use of modified Merrifield chemistry. Ninhydrin assays of the resin plus peptide at each step indicated >99% repetitive yield for each amino acid added. The peptides were then chromatographed on Sephadex G-10 with 30%

acetic acid and lyophilized. The amino acid composition was analyzed at the University of California, Davis, and shown to be within 10% of theoretical values. High-performance liquid chromatography analysis indicated purity in the range of 60 to 80%. Additional peptides (PV-22/23, PV-23/24, VTCG, and VGVRVR) were synthesized by American Peptide Co. (Santa Clara, CA) and were >95% pure. Peptides were stored at −20°C under argon to protect against oxidation, and solutions were prepared immediately before use. The free sulfhydryl group content of solutions of cysteine-containing peptides was routinely tested with Ellman's reagent (Pierce Chemical Co., Rockford, IL) by spectrophotometric assay. Except for PV-22, which showed a tendency to form dimers in solution, the cysteine residues of peptides PV-21, PV-23, PV-22/23, and VTCG remained predominantly (>80%) in reduced form.

11. Plastic microtiter plates (eight-well strips, Costar) were coated with 100 μl of peptides or, as a control, bovine serum albumin (BSA), diluted to 10 μg/ml in 0.1 M carbonate buffer (pH 9.6) and incubated at 4°C overnight. Peptide solutions (1 mg/ml in distilled water) were prepared just before their use in assays. Nonspecific sites were blocked with 1% BSA–phosphate-buffered saline (PBS) for 3 hours at room temperature. Cells were labeled with ^{51}Cr (100 μCi per 10^7 cells/ml) in RPMI 1640 containing 5% fetal bovine serum (FBS) by incubation at 37°C for 1.5 hours. After three washes, cells were resuspended in RPMI 1640 containing 1% BSA (10^6 cells/ml) and 100-μl aliquots were added in triplicate to the microtiter plates. After a 1-hour incubation at 37°C, a Transtar device (Costar) was used to remove the nonadherent cells, and then the places were washed twice with PBS. The individual wells were separated and counted in a gamma counter. Cell lines were obtained from the American Type Culture Association, except for the small-cell lung tumor lines H82 and H446, which were obtained from A. Doyle. All lines were maintained in RPMI 1640 medium supplemented with 10% FBS and subcultivated twice weekly. Experiments were conducted with cells in log phase growth and were repeated at least three times.
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14. The single-letter abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
15. Peptides or CS proteins were labeled in aqueous solution at pH 8.6 with FITC bound to cellulose powder [H. Rinderknecht, *Nature* **193**, 167 (1962)] and then desalted by G-10 or G-25 chromatography. CEM cells (2×10^5 per tube) were incubated with FITC-labeled proteins (50 μg/ml) for 30 min at 37°C, washed, and analyzed with a FACStar Plus flow cytometer (Becton Dickinson).
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29. We thank K. Callahan for expert technical assistance, C.-M. Chuong for helpful discussions, and I. Bathurst of Chiron Corporation (Emeryville, CA) for kindly providing the CS protein preparations. Supported by a contract (DPE-0453-C-00-6060-00) from the U.S. Agency for International Development.

15 March 1990; accepted 9 July 1990

Molecular Analysis of Acute Promyelocytic Leukemia Breakpoint Cluster Region on Chromosome 17

JULIAN BORROW, AUDREY D. GODDARD, DENISE SHEER, ELLEN SOLOMON

Acute promyelocytic leukemia (APL; FAB M3) is characterized by a predominance of malignant promyelocytes that carry a reciprocal translocation between the long arms of chromosomes 15 and 17, t(15;17)(q22;q11.2–q12). This translocation has become diagnostic for APL, as it is present in almost 100 percent of cases. A Not I linking clone was used to detect this translocation initially on pulsed-field gel electrophoresis and subsequently with conventional Southern (DNA) analysis. The breakpoints in ten APL cases examined were shown to cluster in a 12-kb region of chromosome 17, containing two CpG-rich islands. The region is the first intron of the retinoic acid receptor α gene (*RARA*).

ACUTE PROMYELOCYTIC LEUKEMIA (APL) is associated with hypofibrinogenaemia and severe early hemorrhage attributable to the abundance of neoplastic promyelocytes (*1*). Treatment of this attendant coagulopathy necessitates early and accurate diagnosis of the disease. The presence of a consistent chromosomal translocation t(15;17) (*2*) (Fig. 1) in the promyelocytes of the majority of APL patients suggests the location of oncogenic sequences that are rearranged during the leukemogenic process and whose identification would help elucidate the mechanisms of transformation. In other leukemias in which nonrandom translocations have been observed, such as Burkitt's lymphoma (*3*) and chronic myelogenous leukemia (*4*), breakpoint analysis was only possible because there was previous knowledge of the genes that mapped to the breakpoint region. In APL the candidate genes *MPO*, *thra*-1, and c-*erb*B2 have not been reported to be molecularly rearranged (*5*), requiring a broader physical approach to the cloning of the breakpoint.

Translocation breakpoints can be visualized as alterations in rare-cutter fragment sizes on pulsed-field gel electrophoresis (PFGE) (*6*). The use of linking clones (*7*), which span rare-cutter sites, simplifies the detection of these rearrangements (*8*) and helps in the construction of long-range physical maps. In addition, these clones mark the position of a subset of the genes in a defined region (*9*). We constructed a Not I linking library from an interspecies hybrid TRID62 (*10*), which contains 17q as its only human material, to identify Not I sites that flank the APL breakpoint. This library and the sublocalization of 112 human clones over a chromosome 17 mapping panel will be described elsewhere.

The linking clones LCN4A3 (D17S273) and LCN2C11 (D17S163) that were found to flank the APL breakpoint (Fig. 1) were used as probes on PFGE. Unique probes were isolated from both sides of the Not I site and hybridized to somatic cell hybrids containing either the 15q+ or 17q− APL derivative chromosome and to normal controls. Subclone LCN4A3/A (Figs. 2B and 3), which mapped distal to the breakpoint, detected abnormal PFGE band sizes. In Not I digests, the normal fragment of 170 kb was rearranged to 310 kb by the translocation (Fig. 2A). This 310-kb band was the only band detected in two independent 15q+ interspecies hybrids [PJT2A1 (*11*) and PLTI1S (*12*)] constructed from different patients with APL, whereas both the normal and translocated bands were present in bone marrow samples from three other patients (APL 512, APL 511, and APL 510). A sample from patient APL 510 during remission (APL 510-R) did not show the 310-kb band, demonstrating a strong correlation between the genomic rearrangements and the neoplastic state. Single cases of both FAB M1 and FAB M2 leukemias (undifferentiated and myeloblastic acute nonlymphocytic leukemia, respectively) also appeared structurally normal in this region as expected, since the t(15;17) is not associated with these diseases. An extension of the PFGE

J. Borrow, A. D. Goddard, E. Solomon, Somatic Cell Genetics Laboratory, Imperial Cancer Research Fund, London WC2A 3PX, United Kingdom.
D. Sheer, Human Cytogenetics Laboratory, Imperial Cancer Research Fund, London WC2A 3PX, United Kingdom.