sence of other previously characterized HTLV structural proteins in the nonproducer cell line, C81-66-45, provides circumstantial evidence for the importance of p42 in the maintenance of the immortalization in vitro and *trans*-acting transcriptional activation observed in this cell line (*7*). A 38-kD protein, which shares immunochemical properties with p42 and appears to be the LOR region product of HTLV-II, has also been identified in a HTLV-II–immortalized cell line, C3-44/MO (*16*). Further work aimed at characterizing the function of these proteins is likely to yield insight into the transformation-related properties of the HTLV family of retroviruses.

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27 August 1984; accepted 5 September 1984

Identification of the Putative Transforming Protein of the Human T-Cell Leukemia Viruses HTLV-I and HTLV-II

Abstract. *The human T-cell leukemia viruses HTLV-I and HTLV-II are unique among the transforming retroviruses of vertebrates in their ability to transform human T cells in vitro and in their close association with human malignancies (T-cell lymphomas and leukemia). Their genomes are relatively simple, containing the genes* gag, pol, env, *and a 3′ region termed "X." This 3′ region may be responsible for the transforming potential of the viruses. The existence of proteins encoded by the 3′ region has been postulated on the basis of multiple open reading frames. In the present study this region is shown to contain a gene encoding a protein of 40 kilodaltons in HTLV-I and 37 kilodaltons in HTLV-II. It is proposed that these proteins be called, respectively, $p40^{xI}$ and $p37^{xII}$.*

Human T-cell leukemia virus type I (HTLV-I) and type II (HTLV-II) are closely associated with specific human leukemias and lymphomas involving T lymphocytes (*1–4*). Both of these retroviruses transform normal T lymphocytes in vitro, lending credence to their etiologic role in these human malignancies (*5–8*). Most of the known oncogenic retroviruses belong to one of two general groups, depending on the mechanisms by which they induce malignancy (*9*). The long latency of the chronic transforming retroviruses, such as the avian leukosis viruses and the murine mammary tumor virus, is thought to be due to transformation by insertional mutagenesis, a mechanism whereby the proviral genome is integrated at sites near a cellular gene with subsequent activation of the cellular gene (*10, 11*). The second group, known as the acutely transforming retroviruses, carry specific transforming sequences (viral oncogenes) derived from a cohort of normal cellular genes (cellular oncogenes or proto-oncogenes) (*9*). HTLV appears to be unique among the transforming retroviruses in that it does not have a viral oncogene with a normal cellular homolog, nor does it integrate at any preferential sites in tumors or in cells transformed in vitro (*12, 13*). These observations argue against a mechanism involving insertional mutagenesis, and the absence of sequences homologous to normal cellular DNA sequences separate HTLV from the acutely transforming retroviruses.

The nucleotide sequences of HTLV-I and HTLV-II contain a highly conserved region between *env* and the 3′ long terminal repeat (LTR) of the viruses. Such sequences have not been observed in animal retroviruses except for a similar sequence in bovine leukemia virus (*14–17*). This region, termed "X" by Seiki *et al.* (*14*) has no definitely assigned function but is believed to participate in HTLV-induced cellular transformation (*14, 16, 17*). Protein products encoded by this region have not been reported previously; however, the existence of such proteins has been predicted on the basis of four open reading frames (called X-I to X-IV) in HTLV-I (*14*) and three open reading frames (called pX-a to pX-c) in HTLV-II (*16, 17*). Comparison of the nucleotide sequences of the X regions from the two viruses reveals significant sequence homology (about 75 percent) between the X-IV region of HTLV-I and the X-c region of HTLV-II (*16, 17*). The molecular size of the predicted protein encoded by the X-IV region of HTLV-I is 24 kilodaltons if initiation of translation occurs at the first methionine codon of the X-IV region (*14*). Analysis of the sequence, however, reveals that the reading frame remains open for some 100 amino acid codons upstream from the methionine. A similar open reading frame is found in HTLV-II (*16, 17*). This extended open reading frame is sufficient to encode a protein of 39.6 kD in HTLV-I and 36.8 kD in HTLV-II. The high degree of conservation between the HTLV-I and HTLV-II genomes in this region suggested that this reading frame

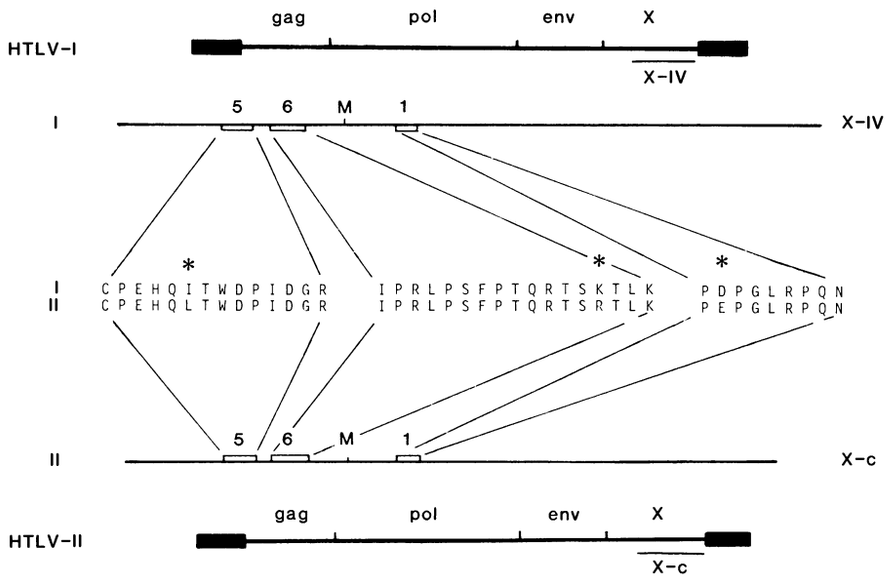

Fig. 1. Schematic representation of the following: the complete HTLV-I and HTLV-II genomes (top and bottom lines, respectively), with the relative positions of the X-IV and X-c regions shown; the X-IV region of the X gene of HTLV-I and the X-c region of the X gene of HTLV-II are immediately below and above their respective genomes, with the relative positions of the synthetic peptides shown. (M marks the position of the first methionine in the X-IV and X-c codon sequence); the amino acid sequence of the synthesized peptides pX-IV-5, pX-IV-6, and pX-IV-1 of HTLV-I and the corresponding amino acid sequence of these peptides in the pX-c region of HTLV-II are shown in the center. Asterisks mark the positions of amino acid differences. The one-letter symbols for the amino acids are A, alanine; R, arginine; N, asparagine; D, aspartic acid; C, cysteine; Q, glutamine; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; and V, valine.

might encode a protein of functional importance, perhaps one involved in cellular transformation. We therefore undertook to identify such proteins by developing specific antisera to potential proteins encoded by this area of the viral genome.

Previous studies have shown that some antigenic determinants of a protein can be represented by short peptides and that antibodies directed against such peptides are frequently of use in identifying the native protein (*18–26*). On the basis of nucleic acid sequence data, the predicted amino acid sequence of individual proteins can be deduced, and oligopeptides with the required amino acid sequences can be synthesized. Such peptides have been useful in generating antisera to a wide variety of proteins, including the transforming gene products of a number of oncogenic retroviruses (*25–35*). We therefore prepared three synthetic peptides termed pX-IV-1, pX-IV-5, and pX-IV-6 (Fig. 1) based on the amino acid codon sequence of the X region of the HTLV-I virus. The peptides were selected from regions in the sequence found to be relatively hydrophilic by the method of Hopp and Woods (*36*) and were synthesized by Peninsula

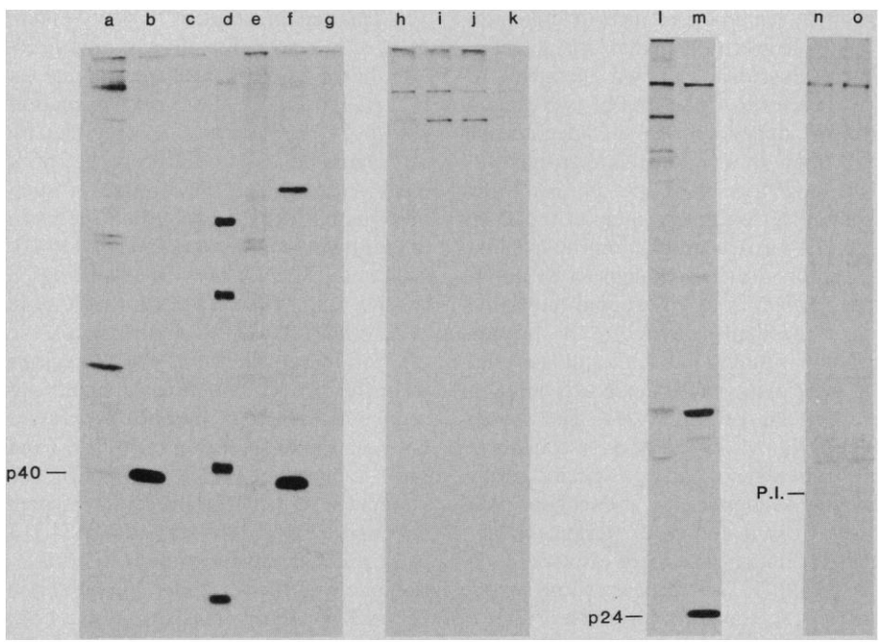

Fig. 2. Immunoprecipitation from various cell lines with antisera directed against peptides derived from the X-IV region of HTLV-I. Indicated lanes are: (lane a) SLB-I cell lysate and unimmunized serum; (lane b) SLB-I cell lysate and antiserum to pX-IV-5; (lane c) SLB-I cell lysate and antiserum to pX-IV-5 which was previously incubated for 30 minutes at 4°C with 10 μl of solution (1 mg/ml) of the pX-IV-5 peptide; (lane d) ^{14}C-labeled protein standard markers (top to bottom: 200 kD, 92.6 kD, 68 kD, 43 kD, and 25.7 kD); (lane e) SLB-I cell lysate and unimmunized serum; (lane f) SLB-I cell lysate and antiserum to pX-IV-6; (lane g) SLB-I cell lysate and antiserum to pX-IV-6 previously incubated with peptide pX-IV-6 as above; (lane h) MOLT-4 cell lysate and antiserum to pX-IV-5; (lane i) HL-60 cell lysate and antiserum to pX-IV-5; (lane j) MOLT-4 cell lysate and antiserum to pX-IV-6; (lane k) HL-60 cell lysate and antiserum to pX-IV-6; (lane l) SLB-I cell lysate and normal human serum; (lane m) SLB-I cell lysate and serum from a patient with adult T-cell lymphoma (HTLV-I–associated), demonstrating immunoprecipitation of p24^{gag} in the infected cells; (lane n) SLB-I cell lysate and unimmunized serum; and (lane o) SLB-I cell lysate and unimmunized serum previously incubated with both the pX-IV-5 and pX-IV-6 peptides. Total cellular proteins were metabolically labeled with [^{35}S]methionine by culturing cells at a concentration of 1×10^6 cells per milliliter in methionine-free Earle's modified minimal essential medium (Flow Laboratories) supplemented with 2 percent glutamine, 10 percent dialyzed fetal calf serum, and [^{35}S]methionine (100 μCi/ml; >600 Ci/mmol; Amersham) at 37°C for 4 to 5 hours. The cells were then washed twice in cold (4°C) phosphate-buffered saline (*p*H 7.4) and lysed at a concentration of 4×10^6 cells per milliliter in radioimmunoprecipitation assay (RIPA) buffer [150 m*M* NaCl, 1 percent sodium deoxycholate, 1 percent Triton X-100, 0.1 percent sodium dodecyl sulfate (SDS), 10 m*M* tris-HCl (*p*H 7.6), and 1 m*M* phenylmethylsulfonyl fluoride (PMSF)]. The cell extracts were clarified by centrifugation at 100,000*g* for 60 minutes at 4°C. The immunoprecipitation reaction mixture consisted of 10 μl of the indicated sera and clarified cell lysate (8×10^6 count/min) in a final volume of 250 μl of RIPA buffer containing bovine serum albumin (2 mg/ml) and 0.07 percent SDS. The antigen-antibody reaction was carried out overnight at 4°C. Immunoprecipitates were collected by addition of 60 μl of a 10 percent suspension of Pansorbin (Calbiochem) for 30 minutes at 4°C. The samples were then washed four times in RIPA buffer and analyzed on 7.5 percent SDS-polyacrylamide gels as described by Laemmli (*47*). Gels were then subjected to fluorography (P.I. indicates comigrating protein seen with unimmunized serum in SLB cells).

Laboratories, Belmont, California. All of the peptides were from areas which are highly conserved in HTLV-II. These peptides were coupled to a carrier protein (keyhole limpet hemocyanin) and were used to generate polyclonal antisera in rabbits as described (*19*). To screen for the putative proteins encoded by the X region, we used lymphoid cells transformed by, or infected with, either HTLV-I or HTLV-II. The criterion used to identify a protein as X-encoded was that the same protein be immunoprecipitated by antisera to at least two peptides representing separate areas of the amino acid codon sequence, thus greatly decreasing the possibility that the immunoprecipitation was due to chance sequence homology between the synthesized peptide and another cellular protein.

The SLB-I cell line is derived from normal human adult peripheral blood cells transformed in vitro with HTLV-I (*37, 38*). A protein of 40 kD was consistently immunoprecipitated from these cells with antisera directed to the pX-IV-5 and pX-IV-6 peptides (Fig. 2, lanes b and f). Immunoprecipitation of this protein could be completely competed away with the relevant peptide, indicating a specific antigen-antibody reaction (Fig. 2, lanes c and g). A faint band of similar migration was detected in SLB cells by unimmunized sera (Fig. 2, lanes a and e), but this protein was not competed away by the specific peptide, indicating a lack of relevance to the immunogenicity of the peptides used (Fig. 2, lanes n and o). Furthermore, the 40-kD protein was not found in control hematopoietic cell lines, including MOLT-4, a transformed T-lymphoblast cell line which is not infected with HTLV, and HL-60, a human promyelocytic cell line (Fig. 2, lanes h and k).

The JLB-I cell line is an HTLV-II–transformed T-cell line that was derived from the normal donor whose cells were used to establish the SLB-I cell line (*7*). In these cells, a protein of 37 kD was found with the same antisera, that is, the antiserum to pX-IV-5 and the antiserum pX-IV-6 (Fig. 3, lanes c and g). Again, immunoprecipitation of this protein could be completely inhibited by the relevant peptide, indicating a specific antigen-antibody reaction (Fig. 3, lanes d and h). The 37-kD protein was not found in control hematopoietic cells that were not infected with HTLV-II (Fig. 2, lanes h to k), nor was it identified by unimmunized sera (Fig. 3, lanes b and f). A second protein of lower molecular weight (approximately 30 kD) was also identified in the JLB-I cell line with antiserum to pX-IV-6 (Fig. 3, lane g). This protein, however, was not recognized by antiserum to pX-IV-5 (Fig. 3, lane c) and its immunoprecipitation was not competed away by peptide (Fig. 3, lane h).

We demonstrated earlier that HTLV-II productively infects B cells and that the virus produced from these cells maintains the ability to transform T cells (*39*). To demonstrate unambiguously the specific association of the 37-kD protein with HTLV-II infection, we examined uninfected and infected cells of the same B-cell line, 729. The 37-kD protein was seen in infected 729 B-cell lysates and not in uninfected 729 cells (Fig. 4, lanes a, b, e, and f). Again, immunoprecipitation of the 37-kD protein found in the HTLV-II–infected B cells could be inhibited by prior incubation of the antisera with the appropriate synthetic pep-

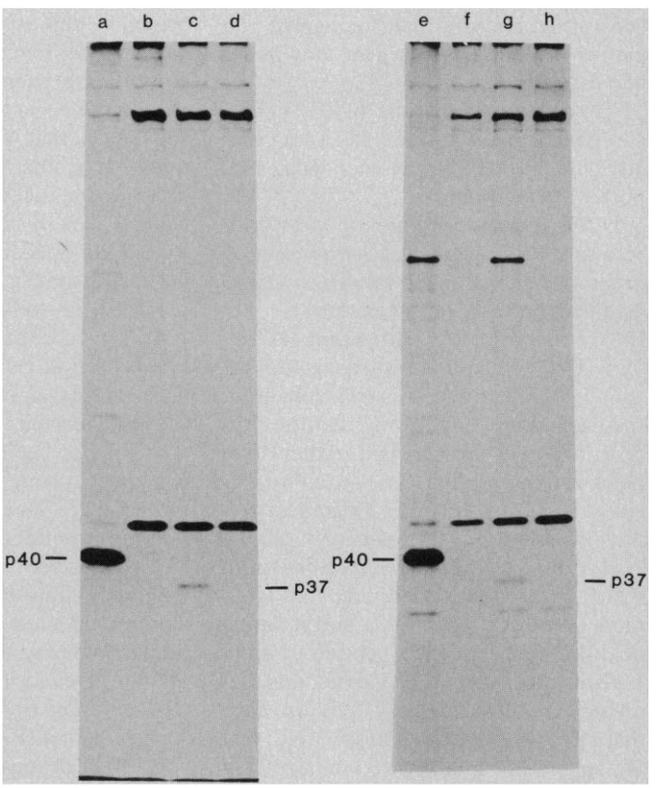

Fig. 3. Immunoprecipitation from cells infected with HTLV-I (SLB-I) and HTLV-II (JLB-I). (Lane a) SLB-I cell lysate and antiserum to pX-IV-5; (lane b) JLB-I cell lysate and unimmunized serum; (lane c) JLB-I cell lysate and antiserum to pX-IV-5; (lane d) JLB-I cell lysate and antiserum to pX-IV-5 previously incubated with pX-IV-5 peptide as described in Fig. 2; (lane e) SLB-I cell lysate and antiserum to pX-IV-6; (lane f) JLB-I cell lysate and unimmunized serum; (lane g) JLB-I cell lysate and antiserum to pX-IV-6; and (lane h) JLB-I cell lysate and antiserum to pX-IV-6 previously incubated with pX-IV-6 peptide.

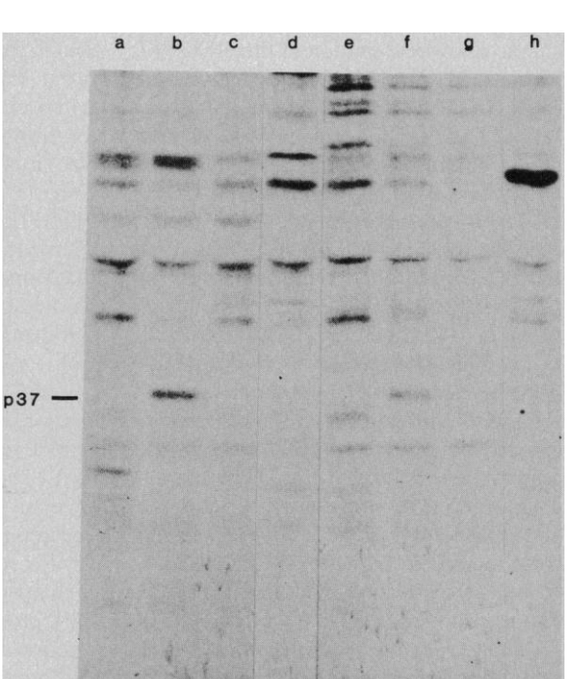

Fig. 4. Immunoprecipitation from uninfected and HTLV-II–infected B cells. (Lane a) 729 (uninfected) cell lysate and antiserum to pX-IV-5; (lane b) 729i (HTLV-II-infected) cell lysate and antiserum to pX-IV-5; (lane c) 729i cell lysate and antiserum to pX-IV-5 first incubated with pX-IV-5 peptide as described in Fig. 2; (lane d) 729i cell lysate and unimmunized serum; (lane e) 729 cell lysate and antiserum to pX-IV-6; (lane f) 729i cell lysate and antiserum to pX-IV-6; (lane g) 729i cell lysate and antiserum to pX-IV-6 previously incubated with pX-IV-6 peptide; and (lane h) 729i cell lysate and unimmunized serum.

tide, and unimmunized sera did not recognize the protein (Fig. 4, lanes c, d, g, and h).

Antisera directed against peptide pX-IV-1 (Fig. 1) failed to recognize any unique protein in cells infected with either HTLV-I or HTLV-II (data not shown). The failure of this serum to recognize the 40-kD or 37-kD proteins may be related to the size of the pX-IV-1 peptide. We have found that shorter synthetic peptides (fewer than ten amino acids) are considerably less effective in generating antisera that recognize the native protein from which they were derived (*40*).

The conclusion that the 40-kD HTLV-1–related protein and the 37-kD HTLV-II–related proteins are X-encoded is supported by the following.

1) The proteins are identified by antisera to at least two peptides representing different regions of the predicted amino acid sequence of the X region.

2) Transformation with either HTLV-I or HTLV-II resulted in recognition of proteins of different sizes in T cells from the same donor, indicating that the proteins were of viral origin rather than being cellular proteins associated with T-cell transformation. The degree of homology between the portions of the HTLV-I and HTLV-II X regions from which the peptides were prepared is almost complete. There is a single amino acid difference in each peptide in comparison with the HTLV-II codon sequence in these areas (*7, 15*). In each instance the change is conservative; that is, isoleucine replaces leucine in pX-IV-5, and lysine replaces arginine in pX-IV-6 (Fig. 1). This degree of homology should allow recognition of the HTLV-II X-encoded protein when peptides based on the HTLV-I X sequence are used. Review of the data, however, clearly shows a difference in the amounts of the 40-kD and 37-kD proteins found in the HTLV-I– and HTLV-II–infected cells, repectively (Fig. 3, lanes a, c, e, and g), even though the same amount of [^{35}S]methionine-labeled cell lysate was used in all immunoprecipitation reactions. This finding may reflect a difference in the relative amounts of the proteins in the infected cells. Alternatively, it may be due to a difference in the avidity of the antisera for the proteins. The amino acid sequences used for the synthesis of the peptides were derived from the codon sequence of the HTLV-I X-IV region rather than the HTLV-II X-c region (Fig. 1). The resulting antisera might therefore be expected to better recognize a protein in which the peptide is conserved with complete fidelity than one in which there are amino acid mismatches.

3) The sizes of the 40-kD and 37-kD immunoprecipitated proteins is close to the sizes predicted from the X regions of HTLV-I (39.6 kD) and HTLV-II (36.8 kD), excluding glycosylation or other posttranslational modifications. Since the antisera recognizing the 40-kD and 37-kD proteins were derived from sequences upstream from the first methionine in the pX-IV open reading frame, we predict that both the 40-kD and 37-kD proteins represent the products of spliced messenger RNA's (mRNA's) consisting predominantly of pX-IV and pX-c sequences, as well as some 5′ viral sequences that supply the methionine initiation codon. Indeed, recent results indicate that the X region of both HTLV-I and HTLV-II is transcribed into a 2.0- to 2.2-kb spliced subgenomic mRNA (*41*). This message would easily accommodate a 37- to 40-kD protein.

4) The fact that the same 37-kD protein is seen in HTLV-II–infected B cells as in HTLV-II–infected T cells, which are transformed, strongly suggests that the 37-kD protein is a virally encoded protein occurring with viral infection and not solely in T-cell transformation. Furthermore, neither the 40-kD nor the 37-kD proteins are found in control hematopoietic cell lines that are transformed but are not infected by HTLV—that is, a human leukemia T-cell line (MOLT-4), a human myeloid leukemia cell line (HL-60), and an Epstein-Barr virus–transformed human B-cell line (729).

It has been suggested that the X region of HTLV is responsible for the transforming potential of the virus (*14, 16, 17, 42*). On the basis of data presented in our study, and in accordance with the accepted convention on naming the putative transforming regions of oncogenic retroviruses (*43*), we propose calling the X-region proteins of HTLV-I and HTLV-II, p40^{xI} and p37^{xII}, respectively.

The function of the *xI* and *xII* genes in HTLV-mediated transformation is not clear. Recent evidence suggests that the region may play a role in regulation of viral RNA expression (*44*). It is possible that the p40^{xI} and p37^{xII} proteins may function in a manner analogous to the papova virus T antigens or the adenovirus EIA proteins. These viral proteins serve an important regulatory role in viral replication as well as being necessary for cell transformation (*45, 46*). The mechanisms by which the p40^{xI} and p37^{xII} proteins might facilitate viral transcription and induce cellular transformation, however, remain uncertain.

With the availability of specific antisera for the *xI* and *xII* products, investigations on the subcellular localization and isolation of the proteins are now possible. Such studies should provide important insights into the role of these proteins in HTLV-induced transformation and disease.

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48. Supported by a grant from Triton Biosciences, by grants CA 32737 and RR 00865 awarded by the National Cancer Institute, and by Cancer Center support grant CA 16042. We gratefully acknowledge the technical assistance of L. Rodriguez, D. Keith, S. Quan, A. Healy, and R. Cortini. We thank L. Kim, B. Colby, and J. Gasson for their advice and encouragement during this study and B. Koers and G. Helfan for their assistance in preparing this manuscript.

30 August 1984; accepted 5 September 1984

Reversal of Knob Formation on *Plasmodium falciparum*–Infected Erythrocytes

Abstract. *The human malarial parasite* Plasmodium falciparum *can produce surface protrusions (knobs) on infected erythrocytes; however, long-term culturing of the parasite results in the appearance of knobless cells. In this study it was found that a knob-producing clone lost the ability to produce knobs in vitro. Furthermore, a clone not producing knobs derived from the knob-producing clone regained the capacity to produce knobby cells in vitro. Certain parasite proteins were associated with the knobby phenotype but not with the knobless type. These results indicate that the parasites change in vitro in a spontaneous and reversible manner independent of immunological selection.*

Isolates of *Plasmodium falciparum* can undergo several changes in culture (*1–5*). Since these isolates probably represent mixed populations when they are placed in culture, subsequent changes may merely reflect preferential growth of subpopulations. Indeed, antigenic differences found in separate isolates obtained from the same geographic area suggest considerable dissimilarity among strains of *P. falciparum* (*6*). Likewise, cloned strains differ from one another and the parental strain in isoenzymes and drug sensitivity (*3, 5, 7*).

Plasmodium falciparum produces knoblike protrusions on infected erythrocytes (*1, 8*) during the trophozoite and schizont stages of development. However, Langreth *et al.* (*2*) cultured knob-producing (K^+) isolates for over 2 years and obtained clones that no longer produced knobs (K^-) (*2*). It appears that the K^- strains originated from cultures that contained mixtures of K^+- and K^--infected cells (*2, 5, 7*).

To determine whether appearance of K^- variants in culture represents a change in vitro or merely the growth of a subpopulation, we cloned a K^+ strain and then screened cultures for infected cells without knobs. K^- parasites from such cells were subcloned and similarly used to select for cells with knobs. Finally, a protein was identified that is present in K^+ but not K^- parasites.

We cultured (*9*) an isolate of *P. falciparum*, strain FVO, obtained from an infected *Aotus* monkey. Before being cloned, the parasite cultures were synchronized for development (*10–12*). Then cells and parasites were grown in 25-cm^3 tissue culture flasks (hematocrit, 2.5 percent; parasitemia, 2 percent) with an appropriate gas mixture (*9*). The flasks were rocked to promote infection of erythrocytes by one parasite each. Fewer than 1 percent of the infected cells contained more than one parasite per erythrocyte, as determined by microscopic observation of Giemsa-stained thin smears. Parasites were cloned in 96-well plates by limiting dilution to an average of one parasite per 100 wells in cultures maintained at a hematocrit of 2 percent. The medium was replaced every 48 hours and fresh erythrocytes were added every 4 days (*3*). After 19 days of growth, stained thin smears were observed and 1 of 192 culture wells was found to contain infected cells. A Poisson distribution of parasites per well was assumed, so that the probability that the parasites in a positive well arose from a single infected cell is $\mu e^{-\mu}/(1 - e^{-\mu})$, where μ is the average density of parasites in the inoculum; μ was determined with the expression $\mu = \ln [1 + (\text{number of positive wells})/(\text{number of negative wells})]$. Thus the calculated probability that the K^+ clone used to select for K^- variants arose from a single parasite is 99.86 percent. Transmission electron microscopy was used to confirm that this clone was present only in knobby cells. On the basis of this observation and statistical analysis of the cloning results, we are reasonably certain that the results presented below were obtained by using a pure K^+ clone.

We used the K^+ clone (Fig. 1A) to select for spontaneously produced K^- variants by means of the following procedure. The K^+ clone was grown in culture and knobless cells were separated from knobby ones in a gelatin-containing medium (*10*) in which knobby cells settled more slowly than knobless ones. The lower phase contained uninfected cells and erythrocytes infected with ring-stage parasites or K^- tropho-

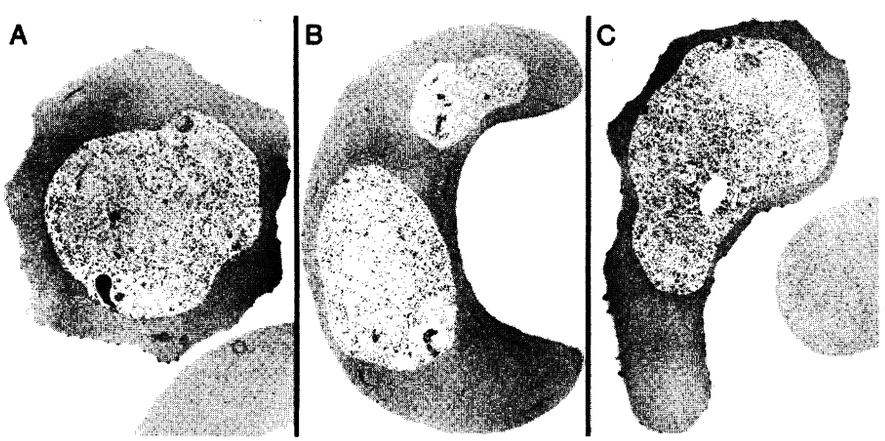

Fig. 1. Electron micrographs of erythrocytes infected with trophozoites of K^+ and K^- strains (×9000). (A) An erythrocyte infected with a K^+ clone isolated from strain FVO. (B) A cell infected with a K^- subclone derived from a mixed K^+-K^- culture after nine gelatin separations. (C) A knobby cell derived from a K^- clone after eight gelatin separations. Cells prepared for electron microscopy (*8*) from synchronized cultures were viewed with a Hitachi HU-12A electron microscope.