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SHORT COMMUNICATION

Sequence Analysis of a Highly Divergent HIV-1-Related Lentivirus Isolated from a Wild Captured Chimpanzee

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Two strains of simian immunodeficiency viruses (SIV) isolated from chimpanzees ($SIV_{CPZ-GAB}$ and $SIV_{CPZ-GAB2}$) originating from Gabon have previously been genetically characterized and shown to belong phylogenetically to the same lineage as the human immunodeficiency virus type 1 (HIV-1). We describe the sequence analysis of a third HIV-1-related virus, $SIV_{CPZ-ANT}$, isolated from a wild captured chimpanzee originating from Zaire. This virus displayed the same genetic organization as HIV-1 and was found to fall on the same lineage as HIV-1 and $SIV_{CPZ-GAB}$. Protein sequence identity with $SIV_{CPZ-GAB}$ ranged from 72% (Pol) to 48% (Env) for the structural proteins, while a particularly divergent Vpu was found (only 25% identity to $SIV_{CPZ-GAB}$). The V3 regions of the SIV_{CPZ} isolates were exceptionally conserved in contrast to the high divergence of V3 among HIV-1 isolates. However, $SIV_{CPZ-ANT}$ did not show a greater degree of sequence similarity with $SIV_{CPZ-GAB}$ than with HIV-1 isolates and represents a quite divergent outgroup of the HIV-1 lineage. Our data suggest multiple introductions of HIV-1 in the human population and shed new light on the origin of the HIV-1 pandemic. © 1996 Academic Press, Inc.

Lentiviruses have been isolated from a variety of primate species and constitute human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2, which cause AIDS) and simian immunodeficiency viruses (SIV) isolated from nonhuman primates. Phylogenetic analysis indicates that these viruses form five major lineages, with the two human viruses falling into different lineages (*1, 2*). HIV-2 is most closely related to SIV_{SM}, isolated from sooty mangabeys, and various lines of evidence suggest that HIV-2 has arisen through cross-species transmission of SIV from sooty mangabeys in West Africa (*3–5*).

The origin of HIV-1 is less clear. Two closely related SIVs, both derived from seropositive chimpanzees in Gabon, have been described. For $SIV_{CPZ-GAB}$, a complete sequence is available (*6*), but information on the second, $SIV_{CPZ-GAB2}$, is limited to 280 bp from the *pol* region (*7*). Although both chimpanzee viruses fall on the same lineage as HIV-1 (*6, 7*), the epidemiological relationships between the human and chimpanzee viruses — and thus the origin of HIV-1 — are uncertain. First, it is not known whether chimpanzees are the natural reservoirs for HIV-1 since only 3/94 animals tested are serologically SIV positive (*8*). In contrast, 30–50% of adult sooty mangabeys in the wild are infected with SIV (*9*). Second, it has been suggested that most SIV (including viruses from chimpanzees, African green monkeys, and sooty mangabeys) may have arisen through cross-species transmission from humans (*10, 11*). Phylogenetic analyses of recently isolated HIV-1 variants reveal that there are two major lineages, named groups M and O: the two Gabonese chimpanzee viruses cluster with group M (*7*) and thus within the HIV-1 radiation. While this relationship would indeed seem to be most simply explained by human-to-chimpanzee transmission, it has been pointed out that cross-species transmission of primate lentiviruses has been frequent enough to undermine the parsimony argument used to make this inference (*12*). A third hypothesis is that both humans and chimpanzees may have acquired lentiviruses from a third species; a number of other African monkey species have been reported to be seropositive for lentiviruses (*13*), but these viruses have yet to be characterized.

We now report on the molecular cloning of a third chimpanzee virus, $SIV_{CPZ-ANT}$, which was previously isolated from a wild captured HIV-seropositive chimpanzee originating from Zaire. The complete $SIV_{CPZ-ANT}$ genome was obtained by polymerase chain reaction (PCR) generating six overlapping genomic fragments. The $SIV_{CPZ-ANT}$ genome has a genetic organization typical for members of the HIV-1 family and encodes nine genes, including *vpu*, which is unique to this lineage. This is consistent

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TABLE 1

Amino Acid Sequence Identity (%) between $SIV_{CPZ-ANT}$, $SIV_{CPZ-GAB}$, and Prototype HIV/SIV Isolates[a]

$SIV_{CPZ-ANT}$ compared with[b]	GAG	POL	VIF	VPR	VPU	TAT	ENV	REV	NEF
$SIV_{CPZ-GAB}$	65	72	52	62	25	57	48	42	51
HIV_{MN} (M)	59	71	54	61	17	61	47	37	47
HIV_{ANT70} (O)	63	70	50	61	19	52	43	57	53
SIV_{AGM}	52	60	26	—	—	34	36	31	43
SIV_{SMM}	51	58	32	46	—	39	37	17	41
SIV_{MND}	51	58	27	48	—	42	31	20	31
SIV_{SYK}	46	54	28	25	—	40	41	34	38

[a] Chromosomal DNA was prepared from human lymphocytes infected *in vitro* with the $SIV_{CPZ-ANT}$ virus isolate. Regions highly conserved between HIV-1 and $SIV_{CPZ-GAB}$ were chosen to design oligonucleotides (and nested oligonucleotides) to prime the reactions. In later experiments, reactions were primed using oligonucleotides derived from the $SIV_{CPZ-ANT}$ sequences themselves. In most cases, the following reaction conditions were used: a 1-min denaturation step at 95°C, a 2-min annealing step at 37°C or 50°C, and 1- to 2-min elongation step at 72°C. For the majority of the regions, a PCR consisting of 30 cycles was insufficient and a second PCR of 30 cycles was needed, using a nested primer pair. The resulting amplification products were tested upon hybridization with HIV-1 probes under low stringency conditions as described (*28*). Sequence analysis was performed on cloned PCR fragments using the dideoxy chain termination method (*29*), and the sequence has been deposited in the GenBank/EMBL database (accession number U42720).

[b] Sequences were aligned using the program Palign (PCgene software package, structure-genetic matrix). The program parameters open gap penalty and unit gap penalty were both set at five. For each alignment, the $SIV_{CPZ-ANT}$ protein sequence was compared individually to the corresponding protein of the following strains: $SIV_{CPZ-GAB}$, HIV_{MN} (representative of group M within the HIV-1 lineage), HIV_{ANT70} (representative of group O within the HIV-1 lineage), $SIV_{AGM\ (TYO)}$, $SIV_{SMM\ (PBJ)}$, $SIV_{MND\ (GB1)}$, and $SIV_{SYK(KEN)}$.

with serological data which also indicate that this virus belongs to the HIV-1 family (*14*). Although we do not know whether the sequence described here represents a biologically functional clone, virus can readily be isolated from the infected animal (*15*). As within primate lentiviruses in general, the proteins encoded by *gag* and *pol* are more conserved than the envelope or regulatory proteins (Table 1). From these protein sequence comparisons, $SIV_{CPZ-ANT}$ is clearly more similar to $SIV_{CPZ-GAB}$ and HIV-1 than to representatives of the other four major lineages but, surprisingly, $SIV_{CPZ-ANT}$ does not seem to be more similar to $SIV_{CPZ-GAB}$ than to HIV-1 isolates. We therefore performed phylogenetic analyses to examine the relationship of $SIV_{CPZ-ANT}$ to other primate lentiviruses. $SIV_{CPZ-ANT}$ was found to fall on the same lineage as HIV-1 and $SIV_{CPZ-GAB}$, but as an outgroup (Fig. 1).

To better appreciate the degree of divergence of $SIV_{CPZ-ANT}$ (Table 1), we investigated the different coding regions and, in particular, the alignments of the *env* and *vpu* sequences. The complete envelope glycoprotein was 686 amino acids long. Of the 15 potential N-linked glycosylation sites (sequons) which are highly conserved within the HIV-1 glycoproteins (*2*), only 11 were conserved in $SIV_{CPZ-ANT}$ compared with 14 in $SIV_{CPZ-GAB}$ (Fig. 2). In addition, an elevated number of cysteine residues was found. Two extra cysteine residues were present at positions 182 and 190 in the V2 domain of the outer membrane protein, while the signal peptide sequence was devoid of the cysteine residue which is highly conserved in HIV-1 and $SIV_{CPZ-GAB}$ (*2*). The distribution of the cysteine residues in the transmembrane part of the protein is remarkable for two reasons: first, compared to $SIV_{CPZ-GAB}$, four extra cysteine residues are present at positions 614, 623, 706, and 788 (Fig. 2A); second, only four cysteine residues are found at equivalent positions when both SIV envelope proteins are aligned (residues 591, 597, 776, and 795). The only SIV subtype currently known to contain a larger number of cysteine residues in its transmembrane protein is SIV_{AGM}, whereas in some primary HIV-1 isolates extra cysteine residues have also been observed (*16*). The structural implications resulting from these changes are presently unknown. The additional cysteine residues in $SIV_{CPZ-ANT}$ might have consequences for folding, and thus for local epitope presentation of the protein, since they are likely to provide extra disulfide bridges (*16, 17*). In accordance with the high sequence divergence found for these envelope proteins, only limited cross-reactivity of HIV-1 antibody-positive sera has been observed with the trans- and outer membrane proteins of $SIV_{CPZ-ANT}$ and, typically, only 1–2% of HIV-1 sera recognize these glycoproteins (*14*). While the complete envelope protein diverges by more than 50% between the two SIV_{CPZ} isolates, only 33% variation is found in the V3 domain (the principal neutralizing domain, PND) which is very low compared with the 80 and 65% divergences in V1 and V2, respectively. The central part of the loop contains the QIGPGMTFYN motif (Fig. 2B), which is identical to the V3 sequence of $SIV_{CPZ-GAB}$, but different from all known HIV-1 V3 sequences (*2*). These results are intriguing since the V3 sequence is hypervariable in HIV-1, whereas the sequences obtained for the two chimpanzee viruses show less divergence compared

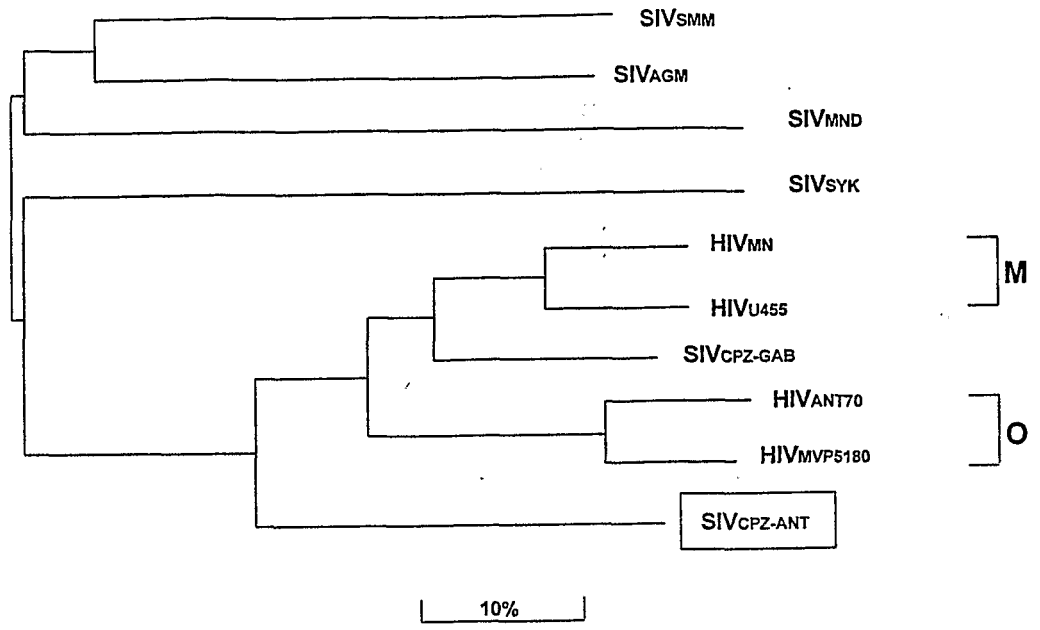
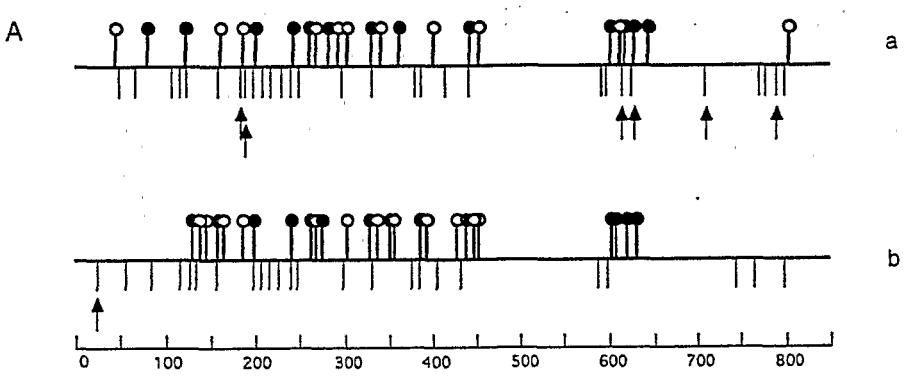


FIG. 1. Phylogenetic relationship of $SIV_{CPZ-ANT}$ to representative primate lentiviruses. Predicted Gag, Pol, and Env protein sequences were aligned and then concatenated; pairwise distances were calculated by Kimura's method (*30*), and the phylogeny was estimated by the neighbor-joining method (*31*) with 1000 bootstraps; these methods were implemented using CLUSTAL W (*32*). The tree is midpoint rooted. Horizontal branch lengths are to scale: the bar indicates 10% (0.10 amino acid replacements per site). All clusters within the HIV-1/SIV_{CPZ} lineage were found in 100% of bootstraps. The same branching order within the HIV-1/SIV_{CPZ} lineage was found in trees estimated from Gag, Pol, and Env proteins analyzed individually and in trees estimated by the maximum parsimony and weighted parsimony methods.



B

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SIVCPZ-GAB          ChRPGNnTrgev---QIGPG----MTFYNiEnvvGDTRsAyC
SIVCPZ-ANT          CiRPGNrTvrnl---QIGPG----MTFYNvEiatGDTRkAfC

HIV-1 A: U455A      CsRPy-NTRknirrysiGsG----qafyvTgkiigdIrqAhC
HIV-1 B: SF2        CtRPnnNTRksi---yiGpG----rafhtTgriigdIrkAhC
HIV-1 D: ELI        CaRPyqNTRqrt---piGlG----qslytT-rsrsiIggAhC
HIV-1 E: CM240      CtRPsnNTRfsi---tiGpG----qvfyrTgdiignIrkAYC
HIV-1 G: VI525      CtRPnnNTRksi---kfGtG----rvlyaTgaiignIrqAhC
HIV-1 O: ANT70      CeRpqIdiqe-m---riGPMaWySM--giggtagnsSRaAYC
HIV-1 O: MVP5180    CiRegIaevqdi---ytGPMrWrSMtlkrsnntsprSRvAYC
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FIG. 2. Envelope protein of $SIV_{CPZ-ANT}$. (A) Representation of $SIV_{CPZ-ANT}$ (a) and $SIV_{CPZ-GAB}$ (b). Potential N-glycosylation sites (NXS or NXT) are indicated with ○ when highly conserved in HIV-1, other sites are designated by ●. The positions of cysteine residues are shown below the line, and arrows indicate cysteine residues unique at that position for each SIV_{CPZ} isolate. The Prosite program of PCgene software was used to localize the potential N-glycosylation sites. Potential glycosylation sites at positions 45 and 125 ($SIV_{CPZ-ANT}$) are followed by a proline residue and are therefore not indicated (*33*). (B) V3 sequence comparison of both SIV_{CPZ} strains (top) and of HIV-1 strains belonging to different subtypes as indicated. The alignment was produced using CLUSTAL W (*32*), with minor manual adjustment. Residues conserved between the two SIV_{CPZ} or within HIV-1 group M or group O are indicated in capital letters; those conserved across all sequences are in bold.

A

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SIVCPZ-ANT        MTNIFEYAFLAFS-IVLWIICIP--ILYKLYKIYKQQQIDNKRNQRI
SIVCPZ-GAB        MTLLVGLVLILVG-LIAWNICIWGYIIKWGYRRYKRHRLETEI-ERL
HIV M             MQALQISAIVGLVVAAIIAIVVW-TIVFIEYRKILRQR---KI-DRL
HIV O             MHHRDLLAIIIISALLFINVILWGFILRKYLEQKEQDRKEREILERL
                  *        .         . .   *.        . .      *.

SIVCPZ-ANT        IEVLSR RLSIDSAIEED ---EE-ADTYY--LGSG--FANPVYREGDE
SIVCPZ-GAB        NLIL-R ERAEDSGNESN GEEEERLEQLIHNYNHNNHFANPMFDL---
HIV M             IDRI-T ERAEDSGNESD GDQEE-LSALV-EMGHHAPWD--INDL---
HIV O             -RRI-R EIRDDSDYESN GEEEQEVMDLV--LSHG--FDNPMFEP---
                              **  *    . *.
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B

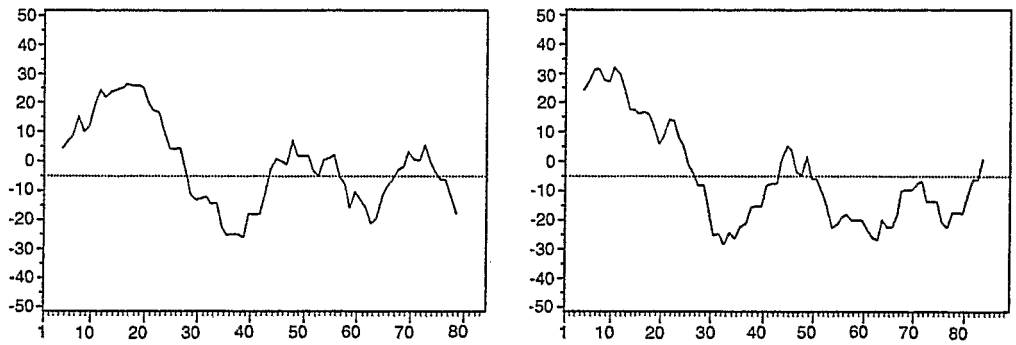

FIG. 3. Vpu protein of $SIV_{CPZ\text{-}ANT}$. (A) Alignment of Vpu proteins from $SIV_{CPZ\text{-}ANT}$, $SIV_{CPZ\text{-}GAB}$, HIV-1 M, and HIV-1 O isolates using CLUSTAL W. Residues identical across all four sequences are indicated by *. The HIV-1 conserved Vpu motif is underlined. (B) Hydropathy profiles of the $SIV_{CPZ\text{-}ANT}$ (left) and $SIV_{CPZ\text{-}GAB}$ (right) Vpu proteins were calculated according to Kyte and Doolittle (*34*). Positive scores are hydrophobic and the amino acid number is indicated on the *X*-axis.

with their complete envelope proteins. The similarity between the two SIV_{CPZ} sequences does not extend beyond the V3 loop and therefore seems unlikely to be due to recombination between these two lineages. Variation is also limited in the V3 sequences of isolates obtained from one infected animal as shown in a study of consecutive isolates obtained from the $SIV_{CPZ\text{-}ANT}$-infected chimpanzee (*15*). Noting that, unlike HIV-1, the V3 domain in SIV_{MAC} from macaques is not involved in neutralization (*18*), these observations raise the question whether the V3 domain in SIV_{CPZ} is functionally equivalent to the PND of HIV-1. The low degree of variation found in the V3 region of HIV-1 viruses upon infection of chimpanzees (*19*) may indicate that these animals react differently to lentivirus infections compared to humans.

The most divergent protein of $SIV_{CPZ\text{-}ANT}$ is the auxiliary protein Vpu. This protein showed little sequence similarity with Vpu of $SIV_{CPZ\text{-}GAB}$ despite its comparable length of 83 amino acids (Table 1). Apart from its position in the genome, *vpu* was identifiable by the hydropathy profile displayed by its deduced protein (Fig. 3). The N-terminal portion is hydrophobic and corresponds to the putative membrane anchor domain as has been suggested for HIV-1 (*20*). Vpu, which is also divergent among HIV-1 variants, usually contains a highly conserved region of 11 amino acids. Only 3 of the 11 residues are conserved in $SIV_{CPZ\text{-}ANT}$, and the residues assumed to be involved in the degradation process of the cellular CD4 receptor are absent (*20*). However, the serine residues probably important for protein phosphorylation are retained, albeit not at the same position (Fig. 2). The WDINDL box which is located at the C-terminus of the protein has also been described as critical for the functioning of Vpu (*21*). This motif is conserved in group M isolates, but not in the SIV_{CPZ} isolates or in group O strains (2). This observation is in keeping with previous data which indicate that proteins with identical or similar functions and structure do not need sharing of extensive sequence similarity (*22*).

In conclusion, as part of an ongoing international effort to document the genetic variation of SIV and HIV, we have studied a new lentivirus, $SIV_{CPZ\text{-}ANT}$, isolated from a wild captured chimpanzee. While this virus clearly belongs to the HIV-1 lineage of primate lentiviruses, it is by far the most divergent representative of this lineage discovered at present. The virus does not cluster with the two previously characterized SIV_{CPZ} isolates, but rather forms an outgroup to all presently known SIV_{CPZ} and HIV-1 isolates. The phylogenetic position of $SIV_{CPZ\text{-}ANT}$ also prompts speculation about the origin(s) of HIV-1 and SIV_{CPZ}. Clearly, it is no longer more parsimonious to suggest that a human virus has been transmitted to chimpanzees (*10, 11*). Furthermore, unless it is argued that $SIV_{CPZ\text{-}GAB}$ was transferred from humans to chimpanzees, it is clear that the two major clusters of HIV-1, groups M and O, must have arisen by two independent introductions into the human population. However, it is not known whether the common ancestor of the entire HIV-1/SIV_{CPZ} lineage has infected chimpanzees. The phylogenetic relationships of the currently known viruses would seem to indicate this, but the low seroprevalence among chim-

panzees argues against it. The alternative remains that both humans and chimpanzees have acquired their lentiviruses from an as yet unidentified third species of African primate. In this scenario, there would also have been two independent introductions into the chimpanzee population. The apparent frequency of cross-species transmission of primate etroviruses (*12, 23*) suggests that this is not unlikely. Exactly when (or where on the phylogenetic tree) these transfers might have occurred remains open to question: thus, whether the $SIV_{CPZ-ANT}$ lineage might have infected chimpanzees only recently, or at a more ancient time near the node connecting that branch to others in the phylogeny, must still be resolved. If it is assumed that a long-term relationship between a pathogen and its host leads to loss of pathogenicity (*24*), then the apparent lack of an AIDS-like pathology in chimpanzees and the failure of HIV-1 to cause disease in chimpanzees (*25, 26*) could be interpreted as an indication that SIV infection of this species is not a recent phenomenon. However, the recent reports of an AIDS-like pathology occurring in an HIV-1-infected chimpanzee (*27*) may require reconsideration of the latter argument.

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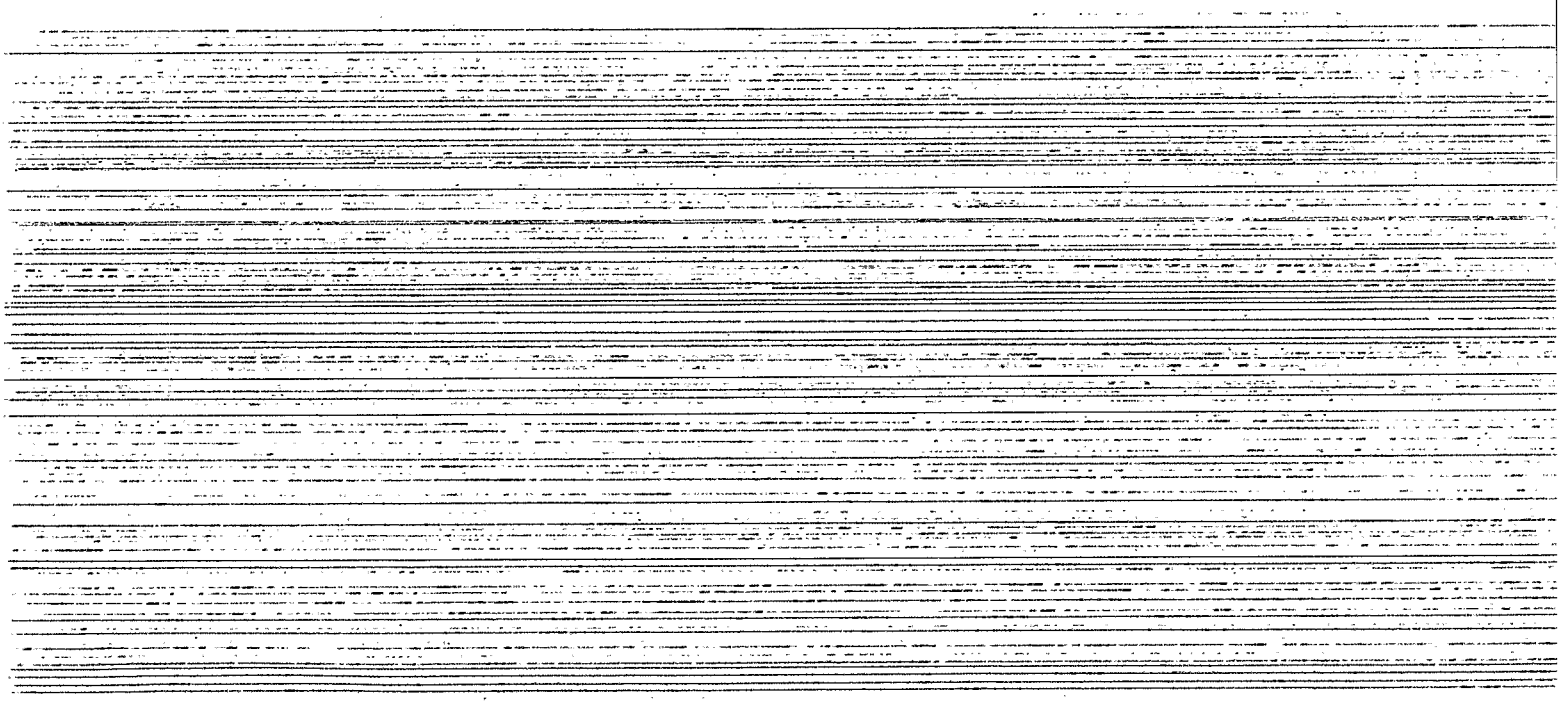
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