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23. Tissues from each mussel were ground in a mortar with a pestle under liquid N_2, and mtDNA was purified as described (*10*). Thorough measures were taken to prevent cross-contamination of tissue or mtDNA samples during all stages of preparation and analysis. After Hind III digestion, fragments were end-labeled with ^{32}P and separated by electrophoresis in 0.8% agarose slab gels (30 V, 12 hours; Fig. 1). DNA was then blot-transferred to a nylon membrane and hybridized to a ^{32}P-labeled probe, synthesized from a cloned *Mytilus* mtDNA template. After hybridization in hybridization buffer (0.36 M NaCl, 0.02 M sodium phosphate, 0.02 M EDTA, 7% SDS, 0.5% nonfat powdered milk, pH 7.7), the membrane was washed four times in 2× SSC (0.30 M NaCl, 0.030 M sodium citrate) and 1% SDS at 65°C for 5 to 10 min; this was followed by two washes in 0.5× standard saline citrate and 0.1% SDS at 65°C for 45 min (Fig. 2). For the digestion, end-labeling, electrophoresis, and Southern blot techniques, we followed procedures described by C. Moritz and W. M. Brown [*Science* **233**, 1425 (1986)].
24. We thank D. Skibinski and R. Koehn for *Mytilus* samples; D. Skibinski for cloned *Mytilus* mtDNA; S. Baldauf, J. Boore, M. Gach, S. Hoffman, R. Hoffmann, T. Jones, N. Klein, C. Moritz, M. Nachman, D. Smith, and L. Vawter for helpful comments; and M. VanBolt and D. Bay for graphics and photography. Supported by collaborative NSF grants (to W.M.B. and to R. K. Koehn) and by an NIH grant (to W.M.B.).

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Isolation of a *rel*-Related Human cDNA That Potentially Encodes the 65-kD Subunit of NF-κB

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A DNA probe that spanned a domain conserved among the proto-oncogene c-*rel*, the *Drosophila* morphogen *dorsal*, and the p50 DNA binding subunit of NF-κB was generated from Jurkat T cell complementary DNA with the polymerase chain reaction (PCR) and degenerate oligonucleotides. This probe was used to identify a *rel*-related complementary DNA that hybridized to a 2.6-kilobase messenger RNA present in human T and B lymphocytes. In vitro transcription and translation of the complementary DNA resulted in the synthesis of a protein with an apparent molecular size of 65 kilodaltons (kD). The translated protein showed weak DNA binding with a specificity for the κB binding motif. This protein-DNA complex comigrated with the complex obtained with the purified human p65 NF-κB subunit and binding was inhibited by IκB-α and -β proteins. In addition, the 65-kD protein associated with the p50 subunit of NF-κB and the κB probe to form a complex with the same electrophoretic mobility as the NF-κB–DNA complex. Therefore the *rel*-related 65-kD protein may represent the p65 subunit of the active NF-κB transcription factor complex.

THE FAMILY OF GENES THAT SHARES sequence similarity with the proto-oncogene c-*rel* encodes a class of DNA binding proteins with diverse regulatory functions (*1, 2*). The recently identified *rel*-related protein p50 is the DNA binding subunit of the NF-κB transcription factor complex (*3, 4*). The NF-κB complex consists of several proteins (*2, 5*) and interacts with the κB DNA sequence, originally identified in the immunoglobulin kappa light chain enhancer (*6*). NF-κB is a pleiotropic transcriptional activator that participates in the induction of numerous cellular and viral genes (*2*). The active complex is formed after dissociation of a 37-kD inhibitory protein IκB. When IκB is released, the active protein complex consisting of a 50-kD polypeptide (p50) and a 65-kD polypeptide (p65) translocates to the nucleus (*7*). Activation of the NF-κB complex is stimulated by several agents including T cell mitogens (*8*), cytokines (*9*), and viral gene products (*10, 11*).

The product of the human T cell leukemia virus (HTLV-1) *tax* gene is a potent activator of NF-κB in T and B lymphocytes (*10, 11*). However, under certain conditions long-term expression of Tax can suppress activation of NF-κB (*12*). NF-κB binding activity represents differential induction of a family of *rel*-related proteins (*13*). We reasoned that the differential responses of long-term Tax expression might reflect alterations in the protein composition of the NF-κB complex. To examine this possibility we sought to identify *rel*-related proteins in the NF-κB complex that were differentially expressed in the presence and absence of Tax. We now report the identification of a cDNA that encodes a *rel*-related protein that appears to be the p65 subunit of the active NF-κB transcription factor complex.

Our approach for identification of *rel*-related gene products consisted of the use of degenerate oligonucleotide primers based on sequences within the 350–amino acid domain conserved between c-*rel* (*14*), *dorsal* (*15*), and the p50 NF-κB subunit (*4*). These primers were used to amplify a 600–base pair (bp) fragment present in cDNA synthesized from mRNA isolated from human Jurkat T and Namalwa B lymphocytes and their respective subclones that express Tax (*12*) (Fig. 1). The amplified fragments obtained by PCR (*16*) were subcloned into a Bluescript expression vector (Stratagene), and 50 inserts were analyzed by DNA sequencing. Of the 50 inserts, 49 were identical and represented a newly discovered gene with significant sequence similarity to c-*rel* (Fig. 1).

One of the inserts was used to screen a cDNA library prepared from Jurkat T cell mRNA. Several clones were identified, and the largest clone, which contained an insert of 2.6 kb, was subjected to further analysis. The first in-frame ATG is at position 80 of the sequence. The nucleotides surrounding this ATG (GCCATGG) conform to the consensus for efficient initiation codons (*17*). This ATG begins an open reading frame of 550 amino acids (Fig. 2), which corresponds to a protein with a predicted molecular size of 63 kD. In vitro transcription and subsequent translation of the cDNA (hereafter referred to as p65 mRNA)

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produced a protein of an apparent molecular size of 65 kD (Fig. 3).

Comparison of the predicted amino acid sequence of this clone with that of other *rel*-related proteins revealed that the similarity to these proteins extends from amino acids 12 to 305. This region of the p65 sequence is about 59% identical to both human and turkey c-*rel* proteins and 43 and 41% identical to *dorsal* and p50 proteins, respectively. A region of v-*rel* shown to function as a nuclear localization signal (*18*) and conserved among the other *rel*-related proteins is conserved in p65 (residues 301 to 304).

Expression of p65 mRNA was examined by Northern (RNA) blot analysis with mRNA from Jurkat T and Namalwa B lymphocytes and clonal lines that express Tax. Each of the cell lines expressed a 2.6-kb species that hybridized specifically to the original PCR-generated fragment (Fig. 4). The abundance of this species was not altered in cells that express Tax. It is therefore unlikely that constitutive expression of Tax influences transcription of p65. Analysis of RNA obtained from nonlymphoid tissue of human, mouse, and simian origin revealed that p65 mRNA is present in each cell line thus far examined (*19*). This probe also hybridized to a 4.0-kb transcript. The p50 subunit of NF-κB is encoded by a transcript of 4.0-kb (*4*). Because of the conserved sequences between p50 and the p65 clone, the 4.0-kb message probably represents cross hybridization between the two sequences.

The molecular size of the 65-kD protein and the sequence similarity to *rel*-related proteins led us to examine whether the cloned protein was functionally related to the 65-kD subunit of NF-κB. Recent studies (*20*) show that the p65 subunit of NF-κB serves as a receptor for the inhibitory subunits IκB-α and -β and exhibits κB-specific DNA binding activity that is considerably weaker than that of the p50 subunit. Ultraviolet cross-linking experiments demonstrated that NF-κB binds to DNA as a heterodimer of the p50 and p65 subunits and that p65 preferentially interacts with the less conserved half-site of κB motifs (*20*). To examine the function of p65, its cDNA was transcribed in vitro with T7 RNA polymerase, and the mRNA was used to program a wheat germ translation lysate. The ability of these translation products to bind to a κB probe was tested by an electrophoretic mobility shift assay (Fig. 5). Translation lysates programmed with p50 RNA showed specific binding to the κB probe (Fig. 5A).

Fig. 2. Deduced amino acid sequence of p65. The predicted amino acid sequence of the longest open reading frame is given. Numbering of amino acids begins with the putative initiation codon. The complete nucleotide sequence has been deposited in GenBank under the accession number M62399.

```
  1  MDELFPLIFPAEPAQASGPYVEIIEQPKQRGMRFRYKCEGRSAGSIPGER  50
 51  STDTTKTHPTIKINGYTGPGTVRISLVTKDPPHRPHPHELVGKDCRDGFY 100
101  EAELCPDRCIHSFQNLGIQCVKKRDLEQAISQRIQTNNNPFQVPIEEQRG 150
151  DYDLNAVRLCFQVTVRDPSGRPLRLPPVLPHPIFDNRAPNTAELKICRVN 200
201  RNSGSCLGGDEIFLLCDKVQKEDIEVYFTGPGWEARGSFSQADVHRQVAI 250
251  VFRTPPYADPSLQAPVRVSMQLRRPSDRELSEPMEFQYLPDTDDRHRIEE 300
301  KRKRTYETFKSIMKKSPFSGPTDPRPPPRRIAVPSRSSASVPKPAPQPYP 350
351  FTSSLSTINYDEFPTMVFPSGRSARPRLGPAPPQVLPQAPAPAPAMVS  400
401  ALAQAPAPVPVLAPGPPQAVAPPAPKPTQAGEGTLSEALLQLQFDDEDLG 450
451  ALLGNSTDPAVFTDLASVDNSEFQQLLNQGIPVAPHTTEPMLMEYPEAIT 500
501  RLVTGAQRPPDPAPAPLGAPGLPNGLLSGDEDFSSIADMDFSALLSQISS 550
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          1                                                  50
Consensus rFRYkCEGrS aGsiPge.St dnnktyPsi. ImnYyG.gkv ritlVTkndp
      p65 RFRYICEGRS AGSIPGERST DTTKTHPTIK INGYTGPGTV RISLVTKDPP
    hcrel RFRYKCEGRS AGSIPQEHST DNNRTYPSIN IMNYYGRGKV RITLVTKNDP
mousecrel RFRYKCEGRS AGSIPGERST DNNRTYPSVQ IMNYYGKGKI RITLVTKNDP
  tkycrel RFRYKCEGRS AGSIPGEHST DNNKTFPSIQ ILNYFGKVKI RTTLVTKNEP
   dorsal VFRYECEGRS AGSIPGVNST PENKTYPTIE IVGYKGRAVV VVSCVTKDTP
NF-kB p50 RFRYVCEGPS HGGLPGASSE KNKKSYPQVK ICNYVGPAKV IVQLVTNGKN

          51                                                 100
Consensus ykpHpHdLVG Kd.Crdgyye aefgper..l .FqNLGIqcV kKkdvkeai.
      p65 HRPHPHELVG KD.CRDGFYE AELCPDRCIH SFQNLGIQCV KKRDLEQAIS
    hcrel YKPHPHDLVG KD.CRDGYYE AEFGNERRPL FFQNLGIRCV KKKEVKEAII
mousecrel YKPHPHDLVG KD.CRDPYYE AEFGPERRPL FFQNLGIRCV KKKEVKGAII
  tkycrel YKPHPHDLVG KD.CRDGYYE AEFGPERQVL SFQNLGIQCV KKKDLKESIS
   dorsal YRPHPHNLVG KEGCKKGVCT LEINSETMRA VFSNLGIQCV KKKDIEAALK
NF-kB p50 IHLHAHSLVG KH.CEDGICT VTAGPKDMVV GFANLGILHV TKKKVFETLE

          101                                                150
Consensus .Ris.g.inp fn........ ........vp .eeqlgdie. ..........
      p65 QRIQTN.NNP FQ........ ........VP IEEQRGDY.. ..........
    hcrel TRIKAG.INP FN........ ........VP .EKQLNDIE. ..........
mousecrel LRISAG.INP FN........ ........VG .EQQLLDIE. ..........
  tkycrel LRISKK.INP FN........ ........VP .EEQLHNID. ..........
   dorsal AR.EEIRVDP FKTGFSHRF. .......... .......... .........Q
NF-kB p50 ARMTEACIRG YNPGLLVHPD LAYLQAEGGG .DRQLGDREK ELIRQAALQQ

          151                                                200
Consensus ...cDLnvVR lcFqvf.lpd ehGnft.alp PvvsnpIyDn rApntaeLrI
      p65 ....DLNAVR LCFQVT.VRD PSGRP.LRLP PVLPHPIFDN RAPNTAELKI
    hcrel ..DCDLNVVR LCFQVF.LPD EHGNLTTALP PVVSNPIYDN RAPNTAELRI
mousecrel ..DCDLNVVR CVFQVF.LPD EDGNFTTAVP PIVSNPIYDN RAPNTAELRI
  tkycrel ..EYDLNVVR LCFQAF.LPD EHGNYTLALP PLISNPIYDN RAPNTAELRI
   dorsal PSSIDLNSVR LCFQVFMESE QKGRFTSPLP PVVSEPIFDK KA..MSDLVI
NF-kB p50 TKEMDLSVVR LMFTAF.LPD STGSFTRRLE PVVSDAIYDS KAPNASNLKI

          201                                    232
Consensus cRvnkncgsv rGgdeIfLLC dKVqKddiev R
      p65 CRVNRNSGSC LGGDEIFLLC DKVQKEDIEV Y
    hcrel CRVNKNCGSV RGGDEIFLLC DKVQKDDIEV R
mouserel CRVNKNCGSV RGGDEIFLLC DKVQKDDIEV R
  tkyrel CRVNKNCGSV KGGDEIFLLC DKVQKDDIEV R
   dorsal CRLCSCSATV FGNTQIILLC EKVAKEDISV R
NF-kB p50 VRMDRTAGCV TGGEEIYLLC DKVQKDDIQI R
```

Fig. 1. Predicted amino acid sequence of the amplified fragment and similarity to the *rel* family of proteins. Complementary DNA synthesized from Jurkat T cell RNA was amplified by PCR with primers specific to domains conserved (*26*) between human (hc*rel*) (*14*), turkey (tkyc*rel*) (*27*), and mouse (mouse*crel*) (*28*), c-*rel*, *dorsal* (*15*), and the p50 (*4*) DNA binding subunit of NF-κB. The underlined amino acids of p65 represent the region that was used for the degenerate oligonucleotide primers. 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.

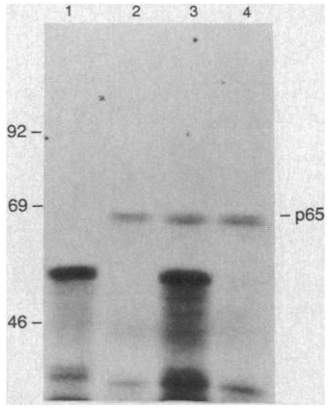

Fig. 3. In vitro translation of p65 mRNA. The p65 cDNA in the Bluescript expression vector was linearized and used as template for an in vitro transcription reaction with T7 RNA polymerase. The in vitro–transcribed RNA was used to program a wheat germ translation lysate that contained ^{35}[S]methionine. Protein products were analyzed on an 8% SDS polyacrylamide gel. For comparison, translation lysates were also programmed with in vitro–transcribed RNA that corresponded to the p50 DNA binding subunit of NF-κB. The p50 cDNA was obtained by PCR with Jurkat T cell cDNA and primers specific to the p50 open reading frame corresponding to amino acids 1 to 502 (*4*). Translation lysates were programmed with lane 1, p50 RNA; lane 2, p65 RNA; and lanes 3 and 4, p50 plus p65 RNA. Molecular size markers (in kilodaltons) are shown at the left of the figure.

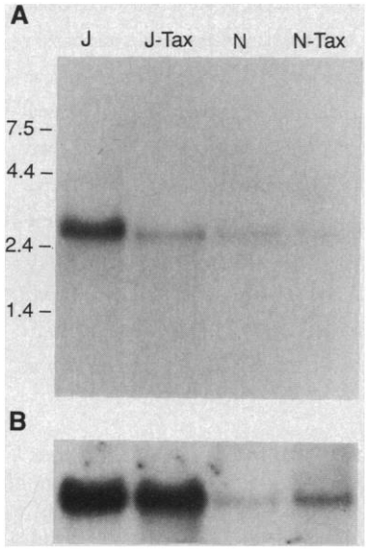

Fig. 4. Northern (RNA) blot analysis of p65 in human T and B lymphocytes. Total RNA was prepared from Jurkat T lymphocytes (J), Namalwa B lymphocytes (N), and clonally derived lines that express the HTLV-1 Tax protein (J-Tax or N-Tax) (*12*). The blot was hybridized with a probe that corresponded to (**A**) the PCR-amplified p65 fragment and (**B**) a probe specific to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to serve as an internal standard. Size markers (in kilobases) are shown at the left of the figure.

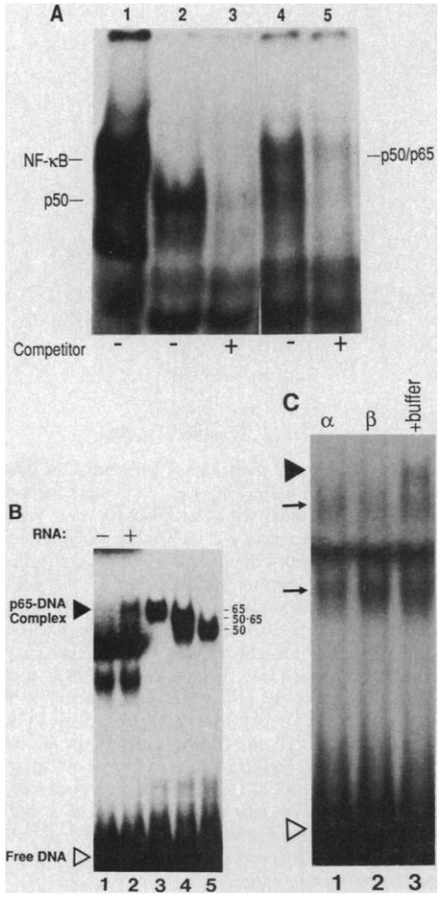

Fig. 5. Properties of in vitro–translated p65 and p50. Electrophoretic mobility shift assays were performed (*29*) with the use of wheat germ translation lysates programmed with p65 or p50 RNA. The κB probes correspond to the binding motif present in the mouse kappa immunoglobulin enhancer. (**A**) Mobility shift assays were performed with lane 1, nuclear extracts from PMA-stimulated Jurkat T cells; lanes 2 and 3, wheat germ extracts programmed with p50 RNA; or lanes 4 and 5, p65 and p50 RNA together. The κB probe was used in all reactions, and competition with cold unlabeled κB probe (15-fold excess) is indicated with a (+). (**B**) Comigration of in vitro–translated p65 with purified NF-κB p65 in mobility shift assays. Binding conditions and purification of proteins from human placenta are described elsewhere (*30*). Mobility shift assays contained κB DNA with lane 1, wheat germ lysate alone; lane 2, in vitro–translated p65; lane 3, purified p65; lane 4, purified NF-κB; and lane 5, purified p50. (**C**) Inhibition of binding of in vitro–translated p65 by IκB-α (*5*) and IκB-β (*31*). Binding reactions were carried out as described above with lane 1, in vitro–translated p65 plus IκB-α; lane 2, in vitro–translated p65 plus IκB-β; or lane 3, IκB buffer alone with in vitro–translated p65. Note that except for p65 no other DNA binding activity (arrows) was affected by the IκB inhibitor proteins.

Using translation extracts programmed with p65 RNA, we observed weak association with the κB probe (*19*). However, by altering the binding conditions (*20*) a p65-κB complex was observed that comigrated with the complex obtained with purified human p65 electrophoretically isolated from NF-κB (Fig. 5B). The complex of p65 and κB DNA migrated slower than that of either the p50-p65 heterodimer (NF-κB) or p50 dimer. To examine whether the 65-kD protein could associate with p50, we cotranslated p50 and p65 RNAs in a wheat germ extract and tested the products in the mobility shift assay. A protein-DNA complex with a slower mobility than that obtained with p50 alone was detected (Fig. 5A). This complex had the same apparent mobility as that of authentic NF-κB present in nuclear extracts prepared from phorbol 12-myristate 13-acetate (PMA)–stimulated Jurkat T cells and was effectively competed by unlabeled κB oligonucleotide.

IκB is the inhibitory protein that stabilizes the cytoplasmic form of NF-κB (*7*). Two forms of this protein have been isolated, IκB-α and IκB-β (*5, 21*). The 65-kD subunit of NF-κB acts as a receptor for IκB. IκB inhibits DNA binding by the p65-p50 heterodimer (NF-κB) but not DNA binding by p50 alone (*20*). To assess whether cloned p65 could interact with the inhibitory subunits IκB-α and -β (*5, 21*), picogram amounts of purified IκB-α and -β were added to the DNA binding reactions with in vitro translated p65. Both IκB forms abolished binding of p65 to DNA (Fig. 5C).

Our data suggest that the 65-kD *rel*-related protein we have identified is the p65 subunit of NF-κB. The ability of p65 to associate with p50 is not surprising, as it shares sequence similarity with p50 within the DNA binding–dimerization domain. Because c-*rel* can also associate with p50 (*3*) and has a similar molecular size of 68 kD, further evidence establishing the identity of p65 as the second subunit of NF-κB was obtained. The 65-kD protein showed weak κB-specific DNA binding activity and the protein-DNA complex showed identical mobility in native gels as purified p65 derived from authentic NF-κB (*20*). The strongest argument that supports the suggestion that the 65-kD *rel*-related protein described herein is the p65 subunit of NF-κB is the inhibition of binding activity by IκB-α. Recent studies show that p65 serves as a receptor for both IκB-α and -β (*20*) and that IκB-α is specific for NF-κB whereas IκB-β can also inhibit c-*rel* DNA binding (*22*). Finally, the partial protein sequence FSQADVHR obtained from the p65 subunit of NF-κB (*4*) is present in our sequence.

Although the p65 subunit of NF-κB shows striking similarity to the *rel* family of proteins, comparison of the first 305 amino acids with other *rel* family members indicates that p65 is more closely related to the *rel* proto-oncogenes than to *dorsal* or p50. There are, however, small distinct stretches of amino acids within this region of p65 that differ from the other *rel* family members. These unique regions of the protein may specify the interaction of p65 with p50 or the IκB repressor proteins. The COOH-terminal 245 amino acids do not show sequence similarity to either *rel* or p50.

Preliminary evidence suggests that the p50 subunit of NF-κB is incapable of transactivating κB-controlled genes (*3*). Because NF-κB is a strong transactivator, the domain or domains necessary for activation are likely to reside in the second subunit, p65. The high degree of sequence similarity between p65 and p50 in the NH_2-terminal 305 amino acids indicates that the COOH-terminal portion of p65, which shares no similarity with p50 (and other *rel* family members), might provide the transactivation domain of NF-κB. This region of the p65 molecule has an unusually high content of proline residues (19%), which is twice that of the *rel*-related region (9.6%). A proline-rich domain (25%) in the COOH-terminus of the CCAAT transcription factor/nuclear factor I is capable of activating transcription when fused to several DNA binding domains (*23*). In addition,

proline-rich regions have been identified in several other mammalian transcription factors including AP-2 (*24*) and c-Jun/AP-1 (*25*). Future studies will likely define the domains of p65 required for DNA binding, dimerization, association with IκB, and transactivation.

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Babbling in the Manual Mode: Evidence for the Ontogeny of Language

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Infant vocal babbling has been assumed to be a speech-based phenomenon that reflects the maturation of the articulatory apparatus responsible for spoken language production. Manual babbling has now been reported to occur in deaf children exposed to signed languages from birth. The similarities between manual and vocal babbling suggest that babbling is a product of an amodal, brain-based language capacity under maturational control, in which phonetic and syllabic units are produced by the infant as a first step toward building a mature linguistic system. Contrary to prevailing accounts of the neurological basis of babbling in language ontogeny, the speech modality is not critical in babbling. Rather, babbling is tied to the abstract linguistic structure of language and to an expressive capacity capable of processing different types of signals (signed or spoken).

A KEY FEATURE OF HUMAN DEVELOPMENT is the regular onset of vocal babbling well before infants are able to utter recognizable words (*1*). Vocal babbling is widely recognized as being continuous with later language acquisition (*2*). The prevailing view is that the structure of vocal babbling is determined by development of the anatomy of the vocal tract and the neural mechanisms subserving the motor control of speech production (*3, 4*). In brain-based theories of language representation, it is argued that the human language capacity has a unique link to innate mechanisms for producing speech (*5*); it has also been argued that human language has been shaped by properties of speech (*6*).

Although there is general agreement that humans possess some innately specified knowledge about language (7), the maturation of the human language capacity may not be uniquely tied to the maturation of speech-specific production mechanisms. Naturally evolved human signed languages exist that are organized identically to spoken languages (for example, phonology, morphology, syntax, and semantics) (*8*). If babbling is due to the maturation of a language capacity and the articulatory mechanisms responsible for speech production, then it should be specific to speech. However, if babbling is due to the maturation of a brain-based language capacity and an expressive capacity capable of processing different types of signals, then it should occur in spoken and signed language modalities.

Hearing infants between 7 and 10 months of age begin to produce a type of vocalization described as reduplicated or syllabic babbling, for example, "dadadada" or "babababa" (*9*). Syllabic vocal babbling is characterized by (i) use of a reduced subset of possible sounds (phonetic units) found in spoken languages (*10*), (ii) syllabic organization (well-formed consonant-vowel clusters) (*11*), and (iii) use without apparent meaning or reference (*12*). Other properties include reduplication, well-defined age of onset, characteristic stages (*12*), and continuity of phonetic form and syllabic type within an individual child's babbling and first words (*2*).

In this study, experimental and naturalistic data were collected from five infants, each videotaped at three ages (approximately 10, 12, and 14 months). Two subjects were profoundly deaf infants of deaf parents (D1 and D2), acquiring American Sign Language (ASL) as a first language. Three control subjects were hearing infants of hearing parents (H1, H2, H3), acquiring spoken language with no exposure to a signed language (*13, 14*).

In studies of vocal babbling, investigators typically transcribe all acoustic forms or sounds produced over a period of time (*15*) and analyze all acoustic forms that are not words to see if they have any systematic organization. If systematic organization is found, the investigator determines whether the organization has phonetic and syllabic features common to spoken languages (*2*).

We analyzed the deaf and hearing infants' manual activities in an identical manner. First, all of the infants' manual activities were transcribed and entered into a comput-

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