Mutations in the First Exon Are Associated with Altered Transcription of c-*myc* in Burkitt Lymphoma

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The c-*myc* proto-oncogene is involved in chromosomal translocations that are specifically and consistently found in Burkitt lymphoma. Although these translocations are thought to lead to a deregulation of c-*myc* expression, the structural and functional basis of this phenomenon has not been identified. Mutations in a specific region spanning approximately 70 base pairs and located at the 3′ border of the first exon of translocated c-*myc* alleles were consistently detected in Burkitt lymphoma cells carrying classic (8:14) as well as variant (8:22 and 2:8) translocations. These structural alterations were accompanied by an altered pattern of c-*myc* transcription, namely, the removal of a block to transcriptional elongation that has been mapped to the same region. Thus, specific c-*myc* mutations leading to the alleviation of this block to transcriptional elongation may represent a general mechanism causing c-*myc* activation in Burkitt lymphoma.

BURKITT LYMPHOMAS (BLs) ARE characterized by specific chromosomal translocations (*1*) that result in the juxtaposition of the c-*myc* locus on chromosome 8 and one of the immunoglobulin (Ig) loci located on chromosomes 14, 22, or 2 (*2*). Although these translocations are thought to lead to the deregulation of c-*myc* gene expression (*3, 4*), definitive evidence for such a phenomenon has yet to be found, and the mechanism involved has yet to be elucidated.

According to one model, regulatory elements of the immunoglobulin loci may act in *cis* to overrule the normal pattern of c-*myc* expression (*4*). However, the known Ig transcriptional enhancer has been found in proximity to c-*myc* in only a minority of BL cases (*5*). An alternative, possibly complementary mechanism is suggested by structural alterations in the 5′ portion of translocated c-*myc* genes (*2, 6, 7*), which may inactivate negative regulatory elements within the first exon and 5′ flanking sequences, thus leading to constitutive oncogene expression. However, as these alterations are heterogeneous and often complex within individual cases (*7*), no common pattern has been identified that would support their functional significance. We have now identified a region at the 3′ border of the first c-*myc* exon that appears to be consistently altered in BL carrying either the most frequent (8:14) or the variant (8:22 and 2:8) translocations. These structural defects are accompanied by functional alterations in the mechanism of c-*myc* transcriptional elongation, the control of which we have recently assigned to the same region (*8–10*).

The region of the translocated c-*myc* allele containing the first exon is consistently altered in BL, either truncated in most sporadic BL (sBL) or mutated in most endemic BL (eBL) (*7*). In particular, a Pvu II site in close proximity to the 3′ border of the first exon is frequently mutated in translocated c-*myc* genes in eBL, most likely reflecting mutations or small rearrangements or both. We have extended this observation to a panel of BLs (Table 1) including cell lines and biopsies representative of (i) the typical 8:14 translocations in eBL carrying breakpoints located at an undefined distance 5′ to the c-*myc* locus, (ii) the subgroup of sBL carrying 8:14 translocations and breakpoints in the 5′ flanking region, and (iii) eBL and sBL cases carrying variant (8:22) translocations and breakpoints located 3′ to the c-*myc* locus. Representative data are shown in Fig. 1 and the results are summarized in Table 1. In 15 of 26 cases, including 10 of 13 eBL biopsies, a Pvu II polymorphism involved a single allele of the 0.8-kb Pvu II fragment spanning the first c-*myc* exon and was caused by nucleotide changes in the 3′ Pvu II site (Fig. 1). The Pvu II mutation is found in the tumor cells (BL60 in Fig. 1) but not in control cells (LCL261) obtained from the same individual or in normal cells from a panel of normal individuals (*7*).

To confirm and extend these results, we used a ribonuclease (RNase) protection assay capable of detecting single-base mismatches between an in vitro transcribed antisense RNA probe and the corresponding cellular RNA (*11*). By means of a normal c-*myc* probe spanning a 72-bp region across the Pvu II site and a control probe spanning first intron and second exon c-*myc* sequences (Fig. 2), we have analyzed RNA from a panel of BL cell lines including all the Pvu II–negative cases. In 7 of 12 BL cases the 72-bp band either disappeared (indicating the presence of multiple mutations) and/or was substituted by subfragments (indicat-

Table 1. Alterations of c-*myc* first exon (Pvu II region) in BL. Note that in Ramos cells the nucleotide sequence of the translocated c-*myc* gene has an A-T mutation 6 bp downstream from the Pvu II site (*15*); in KK124 cells a 28-bp deletion 57 bp upstream of the Pvu II site has been reported (*26*); in MC116 cells a mutated Hae III site 212 bp upstream of Pvu II has been detected (*7*), while the Pvu II region may be either normal or mutated with changes undetectable by the RNase assay; in BL02 cells mutations are found by nucleotide sequence analysis of the exon 1; one mutation was found 98 bp 5′ to the Pvu II site (*27*). For complete nucleotide sequence of translocate c-*myc* alleles in the Daudi and Raji cell lines see (*28, 29*).

BL cases	Type	Translocation	Breakpoint location*	Mutation†	
				Pvu II	RNase
Cell lines					
Ramos	Sporadic	8:14	5′ flanking	−	−
MC116	Sporadic	8:14	5′ flanking	−	−
CW678	Sporadic	8:14	5′ flanking	−	+
EW36	Sporadic	8:14	5′	ND	+
Daudi	Endemic	8:14	5′	+	+
P3HR1	Endemic	8:14	5′	+	+
AG876	Endemic	8:14	5′	−	+
Raji	Endemic	8:14	5′	+	ND
PA682	AIDS-related	8:22	3′	−	+
NAB9b	Sporadic	8:22	3′	+	−
BL60	Endemic	8:22	3′	+	+
BL02	Sporadic	8:22	3′	−	−
KK124	Sporadic	8:22	3′	−	−
Biopsies					
	Endemic	ND	5′ or 3′	10/13	ND

*Location relative to the c-*myc* locus; 5′ flanking indicates sequences within 2 kb 5′ to the first exon. †Pvu II: mutations detected (+) or not detected (−) in the first exon Pvu II site by Southern blot hybridization. RNase: mutations detected (+) or not detected (−) by the RNase protection assay with the ScrFI probe (Fig. 2). ND, not done.

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ing the presence of one or few mutations) (Fig. 2). Positive cases included BL cases (cell lines AG876, CW678, and PA682) that scored negative according to the Pvu II assay (Table 1). Note that one Pvu II–positive case (NAB9b) was not positive in the RNase protection assay. This is expected for some cases, because some of the base transitions, in particular U-U and possibly G-U mismatches, are not recognized by this assay (*11*). Four BL cell lines (Ramos, MC116, KK124, and BL02) were negative in both Pvu II and RNase protection assays. However, in three of these cases mutations within a 100-bp range from the Pvu II site were detectable by nucleotide sequencing and in the fourth case a mutation was detected within a 212-bp range by restriction enzyme analysis (Table 1). The fraction of positive cases (10 of 13) among the tumor biopsies is a minimal estimate, because RNase analysis could not be performed on those cases. We conclude that (i) structural alterations of the c-*myc* gene are detectable in 23 of 26 BL cases representative of the various types of translocations and, notably, in 23 of the 23 cases for which a combination of methods was used; (ii) in at least 17 of these 23 cases mutations were found within the 72-bp region containing the Pvu II site.

Structural alterations of this same genomic region are recognizable in the nucleotide sequences of all known translocated c-*myc* genes, including the c-*myc* genes from cell lines BL22, JBL2, and LY67, which carry (8:14), (2:8), and (8:22) translocations, respectively (*12*). Most notably, mutations in the same region have been reported for a c-*myc* oncogene displaying 3′ rearrangement in a case of multiple myeloma (*13*). These observations suggest that these mutations may represent a general mechanism for c-*myc* activation resulting from different rearrangements in different types of tumors.

We reported a block to transcriptional elongation near the 3′ end of exon 1 in the wild-type human c-*myc* gene that results in an excess of exon 1 as compared to intron 1 and exon 2 transcription (*8*). This block is responsible for the decreased transcription of c-*myc* in differentiated myelomonocytic cells (*8, 9*) and has also been observed in murine cells (*10*). In addition, the increase in c-*myc* RNA observed when quiescent human tonsillar B cells are activated with different mitogens is due primarily to the release of this block to transcriptional elongation (*14*). The observation that 3′ exon 1 sequences are either separated from the rest of the gene or mutated in BL suggests that these alterations may play a role in activation of the c-*myc* gene by disrupting the premature termination of transcription that normally occurs near the end of exon 1. To test this hypothesis, we analyzed the nuclear runoff transcription of the c-*myc* gene in peripheral blood lymphocytes (PBL) immortalized by Epstein-Barr virus (EBV) and in three classes of BL cells that retain the first c-*myc* exon (Table 1): (i) sBL carrying breakpoints in the 5′ flanking region (Ramos and CW678); (ii) sBL or eBL carrying variant (8:22) translocations with breakpoints 3′ to the c-*myc* locus (BL60 and NAB9b); and (iii) eBL carrying 5′ distant breakpoints (Daudi and P3HR1). In contrast to the excess of exon 1 over exon 2 transcription found in EBV PBL, in all the BL lines there was equivalent transcription of exon 1, intron 1, and exon 2, indicating a lack of elongation block in the exon 1–intron 1 region (Fig. 3). Although no elongation block is observed in BL60 cells, the elongation block was present in control cells from the same individual (LCL261). We have also observed equimolar nuclei runoff transcription of exon 1 and exon 2 in BL cell lines KK124, AG876, and BL02 (*14*).

The correlation between structural and functional alterations mapping to the same region in translocated c-*myc* genes in BL suggests a link between the two phenomena, that is, that mutations may cause the release

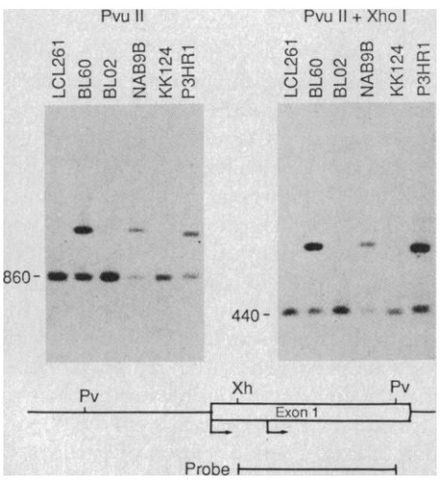

Fig. 1. Mutations in the Pvu II site of translocated c-*myc* alleles in BL. DNAs were digested with Pvu II or Pvu II–Xho I as indicated and analyzed by Southern blot hybridization with the exon 1–specific c-*myc* probe shown. The double digestions show that the 3′ Pvu II is the one mutated in BL60, NAB9b, and P3HR1 DNA. The size of germline bands is indicated. LCL261 is an EBV PBL derived from normal B cells from the same patient from whom the BL60 line was derived. Pv, Pvu II; Xh, Xho I. Arrows indicate the two major c-*myc* promoters (*28*).

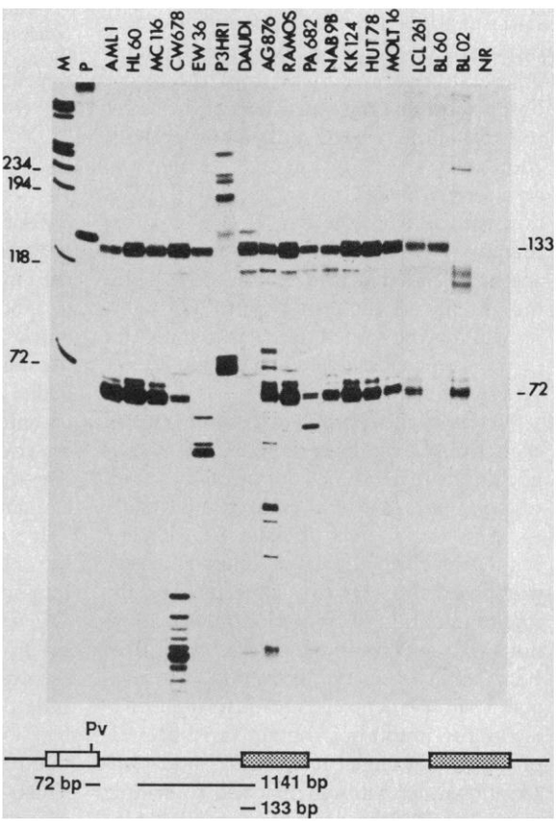

Fig. 2. Mutations in the Pvu II region of c-*myc* exon 1 detected by RNase protection assay in BL. Total cellular RNA was hybridized as in (*11*) (except for the hybridization temperature, which was 42°C) to a uniformly labeled antisense RNA transcript [generated by SP6 promoters in pGEM vectors (Promega Biotec)] corresponding to the c-*myc* regions illustrated at the bottom. The amount of RNA used for hybridization varied from 2 to 100 μg according to the amount of c-*myc* expressed by each individual cell line. After hybridization with first exon (72 bp) and second exon (1141 bp) probes, samples were digested with RNase A and T1 at 37°C for 2 hours (*10*) and then loaded on a 10% polyacrylamide, 8*M* urea gel. The 72-bp probe is representative of a 72-bp ScrFI fragment spanning from 34 bp 5′ to 38 bp 3′ to the Pvu II site (*28*). The 1141-bp probe spans the region from the Xba I site (5′) to the Pst I site (3′) (*28*) and protects a 133-bp fragment in the case of a normally spliced exon 2. The open box represents c-*myc* exon 1; the shaded boxes represent c-*myc* exons 2 and 3. Markers (M) are Hae III fragments end-labeled with ^{32}P from ϕ-χ 174 DNA. Probes (P) appear larger than the expected sizes owing to the presence of polylinker sequences in the pGEM vectors. NR, no cellular RNA added to hybridization. AML1, HL60, HUT78, MOLT16, and LCL261 are non-BL cell lines used as controls.

of the block of transcript elongation. It is possible that a single point mutation may be sufficient for abrogating the transcriptional block. In fact, although some of these cell lines contain c-*myc* genes with multiple mutations (for example, Daudi) (*6*), in Ramos cells the only mutations within the activated allele occur 6 bases downstream of the Pvu II site in exon 1 and 158 bases upstream of the P1 cap site in the 5′ flanking sequences (*15*). The identification, however, of a few other cases (KK124, MC116, and BL02) carrying mutations or deletions located more distantly from the frequently affected Pvu II region (Table 1) suggests that the functional domain involved in the control of elongation may extend further 5′ or 3′ or that other functional domains may occasionally be involved.

One alternative interpretation of our findings is that the differences in the patterns of c-*myc* transcription observed in BL and EBV PBL cells may reflect physiologic differences in c-*myc* regulation in different cell types, rather than the presence of mutations in the translocated c-*myc* alleles in BL. We consider this hypothesis unlikely because BL cells and EBV-infected PBL represent B cells at the same or very similar stage of differentiation (*16*). In addition, we have recently shown that the transition from the immortalized status of EBV PBL to the fully transformed status of BL is dependent upon constitutive expression of the c-*myc* oncogene (*17*). Another explanation derives from the recent observation that the translation of the larger (67 kD) of the two (67 and 64 kD) proteins encoded by the c-*myc* locus (*18*) is initiated at a cryptic (CTG) site located 9 bases downstream from the Pvu II site, and that this form of the c-*myc* protein is either absent or mutated in most BL (*19*). Thus, the absence of the wild-type 67-kD c-*myc* protein may be related to the altered control of transcript elongation and to the pathogenesis of BL.

We have also observed that the rate of exon 1 transcription in these BL cell lines is not different from the rate of exon 1 transcription in EBV-immortalized PBL (Fig. 3). This is also true for the BL cell line Manca in which the Ig enhancer is closely juxtaposed to c-*myc* (*8*). Thus, altering the rate of initiation of transcription of c-*myc* is not a critical event in the generation of BLs. Furthermore, because different BL cases display a similar degree of read-through of c-*myc* transcription but contain variable levels of c-*myc* messenger RNA (*20*), post-transcriptional mechanisms may also contribute to the levels of steady-state c-*myc* RNA in BL. However, the absolute levels of steady-state c-*myc* in RNA may not be as critical in the pathogenesis of BL as the loss of the capacity to regulate c-*myc* expression via transcriptional elongation. The presence of the block to c-*myc* transcriptional elongation in quiescent B cells and the release of this block in activated B cells (*14*) raises the possibility that one component in the pathogenesis of BL could be the failure of BL cells to enter quiescence or differentiation as a result of the inability of these cells to regulate c-*myc* at the level of transcriptional elongation.

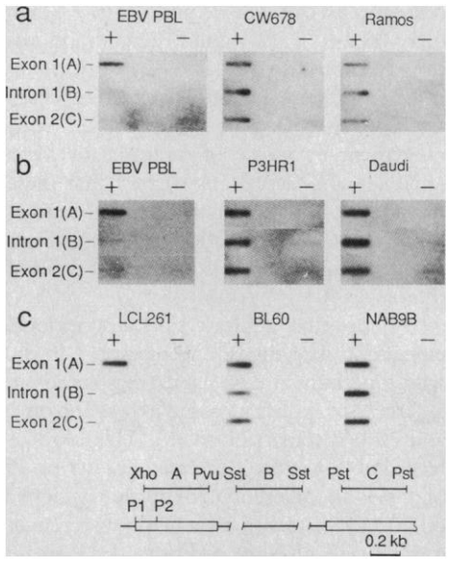

Fig. 3. Nuclear runoff transcription of c-*myc* in lymphoblastoid and BL cell lines. Analysis of (**a**) sBL with breakpoints in the c-*myc* 5′ flanking region; (**b**) eBL carrying 5′ distant breakpoints; and (**c**) variant translocations (8:22) with 3′ breakpoints (Table 1). The total input [^{32}P]RNA (15×10^6 cpm/ml) was identical for the cell types within (a), (b), or (c); thus, hybridization to any particular probe (for example, exon 1) is directly comparable among the three samples in any particular group. Preparation and RNase pretreatment of nuclei, the nuclear runoff procedure, and hybridization and washing conditions are as described (*7*), except that in (c) (and all subsequent experiments) GeneScreen plus (New England Nuclear) rather than nitrocellulose was used for the slot blots of single-stranded M13 DNA and, consequently, SDS was increased to 1% in the hybridization buffer. + and − refer to probes that detect sense and antisense transcripts, respectively. Lengths of probes (in nucleotides): (a) 443; (b) 606; and (c) 414.

It remains to be established whether other factors, such as the influence of putative transcriptional enhancers from Ig loci or alterations in the chromatin structure caused by the chromosomal translocation, are also involved in the activation of c-*myc* in BL. Indeed, truncated c-*myc* genes lacking the first exon do not lead to an increase in frequency of tumors in transgenic mice (*21*). Thus, the simple removal of the region involved in the block of transcriptional elongation may not be sufficient for activation in the absence of sequences responsible for the maintenance of high levels of transcriptional initiation. However, mutated nontruncated c-*myc* alleles most likely retain the regulatory elements that mediate transcriptional induction (*22*). Thus, these alleles may behave as constitutively expressed, activated oncogenes in the absence of a negative control of transcriptional elongation. In addition, the presence of Ig sequences, even at remote distances to c-*myc*, may also be necessary for c-*myc* activation by preventing the formation of the inactive c-*myc* chromatin pattern observed during terminal hematopoietic cell differentiation (*23*). These questions can be addressed by testing whether mutated c-*myc* alleles are tumorigenic in vivo in transgenic animals or in vitro in EBV-infected lymphoblasts in the presence or absence of linked Ig sequences (*17*).

Finally, the possibility that single base pair mutations in the Pvu II domain may contribute to c-*myc* deregulation implies that additional mechanisms of c-*myc* activation may exist that can be independent of chromosomal translocations with Ig loci. Recombination per se, that is, other cytogenetically undetectable genomic rearrangements, may predispose a chromosomal domain to mutation, as suggested by the presence of mutations in activated c-*myc* alleles adjacent to proviral integration sites in avian B cell lymphomas (*24*). In addition, mutations may occur in the absence of chromosomal recombinations as a consequence of chemical or physical mutagenesis, as in the case for the c-*ras* genes (*25*). Thus, c-*myc* activation may represent a more frequent event in tumorigenesis than presently suspected.

Note added in proof: R. LeStrange and M. Groudine have recently observed equimolar transcription of c-*myc* exon 1, intron 1, and exon 2 in a BL cell line in which the translocated c-*myc* allele is normal in exons 1, 2, and 3. However, this allele contains a deletion in the 5′ region of intron 1, where we previously mapped a hypersensitive site associated with the block to elongation (*8*). This hypersensitive site is missing in this BL cell line. This result, in combination with the data presented in this report, indicates that the domain responsible for the block to elongation includes sequences in the 3′ portion of exon 1 and 5′ region of intron 1.

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30. We thank I. Magrath, P. Levine, G. Lenoir, and R. Eisenman for providing us with most of the cell lines and tumor biopsies. Supported by NCI grants 37165 and 37195 to R.D.F. and 28151 to M.G. and by NSF grant DCB 8502591 to M.G. R.D.-F. is a Scholar of the Leukemia Society of America.

29 April 1987; accepted 4 August 1987

Acylation of Proteins with Myristic Acid Occurs Cotranslationally

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Several proteins of viral and cellular origin are acylated with myristic acid early during their biogenesis. To investigate the possibility that myristylation occurred cotranslationally, the BC_3H1 muscle cell line, which contains a broad array of myristylated proteins, was pulse-labeled with [^{3}H]myristic acid. Nascent polypeptide chains covalently associated with transfer RNA were isolated subsequently by ion-exchange chromatography. [^{3}H]Myristate was attached to nascent chains through an amide linkage and was identified by thin-layer chromatography after its release from nascent chains by acid methanolysis. Inhibition of cellular protein synthesis with puromycin resulted in cessation of [^{3}H]myristate-labeling of nascent chains, in agreement with the dependence of this modification on protein synthesis in vivo. These data represent a direct demonstration that myristylation of proteins is a cotranslational modification.

A VARIETY OF VIRAL AND CELLULAR proteins has been shown to be modified by the covalent attachment of the 14-carbon saturated fatty acid, myristate, linked through an amide bond to glycine at their amino termini (*1*). Two of the most thoroughly studied myristylated proteins are the transforming protein of Rous sarcoma virus, $p60^{v\text{-}src}$, and the proto-oncogene product, $p60^{c\text{-}src}$. These polypeptides are translated on free polysomes and myristylated before being transported to the plasma membrane (*2–4*). Deletion or modification of the first 14 NH_2-terminal amino acids of $p60^{v\text{-}src}$ does not influence intrinsic tyrosine kinase activity, but prevents myristylation and membrane association, and abolishes the transforming activity of the protein (*3–7*). Together, these observations suggest an important role for myristylation in targeting proteins to the plasma membrane and in cellular transformation.

Inhibition of protein synthesis results in immediate cessation of myristylation of cellular and viral proteins, indicating that this covalent modification is an early processing step, tightly coupled to translation (*2, 8–10*). Studies with inhibitors of protein synthesis cannot determine, however, whether myristate attachment occurs cotranslationally or within seconds after completion of polypeptide synthesis. To define precisely when myristate is attached to newly synthesized acylproteins, we have examined whether nascent polypeptide chains are modified by myristic acid.

To obtain nascent chains, BC_3H1 cells were labeled with [^{35}S]methionine for 10 minutes, cell extracts were prepared, and polyribosomes were isolated by magnesium precipitation (*11*). Ribosome pellets were solubilized and were applied to a QAE-Sephadex anion-exchange column in buffer containing 0.1*M* NaCl. This technique, which was adapted from the method described by Cioli and Lennox (*12*), allows isolation of nascent polypeptide chains because of binding of the RNA component of peptidyl transfer RNA (tRNA) to the column. At low salt concentrations, mature polypeptides are not retained by the column, whereas the negatively charged tRNA moiety attached to nascent chains binds to the column. Nascent chains covalently associated with tRNA elute when the salt concentration of the column buffer is increased to 1.0*M* (Fig. 1A).

A typical fractionation on QAE-Sephadex of a ribosomal pellet isolated from [^{35}S]-methionine-labeled cells is shown in Fig. 1, A and C. The large amount of radioactivity that passes through the column in 0.1*M* NaCl represents completed proteins associated with the ribosomal pellet. Increasing the salt concentration to 1.0*M* NaCl resulted in elution of a peak of [^{35}S]methionine-labeled material. To demonstrate that polypeptides contained in this high salt fraction were retained by QAE-Sephadex through a covalent interaction with RNA, we treated ribosomal pellets with 0.3*N* NaOH or ribonuclease (RNase) before fractionation (Fig. 1, B and D, respectively). Each treatment eliminated more than 95% of the [^{35}S]methionine-labeled material previously retained by the ion-exchange resin, demonstrating that RNA was required for its association with the column.

The specificity of the fractionation procedure for isolation of nascent chains was assessed further by diluting an aliquot of the high salt fraction to the low salt concentration of the starting buffer and reapplying the sample to a new column (Fig. 1E). Approximately 80% of the radioactivity was retained by the second column, thereby demonstrating that the high salt fraction was not contaminated with mature polypeptides. In addition, material that passed through the original column did not bind when reapplied to a new column (Fig. 1F), which

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