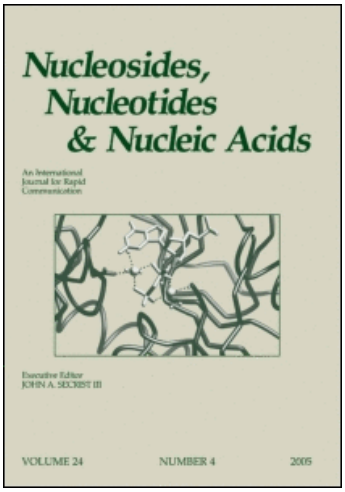

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Kanehara, H. , Wada, T. , Mizuguchi, M. and Makino, K.(1996) 'Influence of a Thiophosphate Linkage on the Duplex Stability - Does Sp Configuration Always Lead to Higher Stability Than Rp?', Nucleosides, Nucleotides and Nucleic Acids, 15: 6, 1169 — 1178
To link to this Article: DOI: 10.1080/07328319608007385
URL: http://dx.doi.org/10.1080/07328319608007385

NUCLEOSIDES & NUCLEOTIDES, 15(6), 1169-1178 (1996)

INFLUENCE OF A THIOPHOSPHATE LINKAGE ON THE DUPLEX STABILITY - DOES Sp CONFIGURATION ALWAYS LEAD TO HIGHER STABILITY THAN Rp?

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ABSTRACT: In order to design an oligodeoxynucleoside phosphorothioate as an antisense molecule, it is important to establish the structure of the S-oligo with a strong affinity to the target RNA. In these molecules, internucleotide thiophosphate linkages produce diastereomers, the number of which increases in proportion to 2^n (n: number of thiophosphate). To estimate the effect of this linkage on the duplex stability by UV melting curves, oligodeoxynucleotides having a single thiophosphate (referred to S-oligo), dGCNsN'CG (s: thiophosphate, N, N' = A or T), were prepared and their diastereomers isolated by HPLC. As demonstrated previously, the melting temperatures (Tm) for the Sp isomers were higher than those of the Rp when DNA was a target. On the other hand, it was found that for RNA as a target, the Rp isomers of dGCTsTCG and dGCAsTCG had higher stability than the Sp, and that the difference in the Tm values between the diastereomers was smaller than when DNA was a target. With dGCsTsACG, which has two thiophosphates, it was also found that the Tm values decreased with an increase in the number of thiophosphate linkages, and that the difference in Tm between the diastereomers was smaller when RNA was a target. Consequently, in practical clinical applications where RNA is a target, the influence of thiophosphate chirality on the duplex structure is almost negligible and Rp/Sp separation of an S-oligo may be of no major concern.

The principle of the antisense method is to control specific gene expression by using complementary DNA against the target unmodified DNA (DNA) or mRNA. This method employed DNA as an antisense molecule in the initial studies.[1] However, this molecule is rapidly decomposed by nucleases, hence reducing its gene regulatory ability.[2] Therefore, oligomers with a variety of modified internucleotide linkages were developed: the methylphosphonate[3] linkage was first used, but had several weak points, in particular an inability to induce RNaseH activity. Among other such oligonucleotide analogs, the oligodeoxynucleoside phosphorothioate linkage was found to have the largest capability to overcome these weak points, and oligomers containing it have been reported to have an effective inhibitory activity against HIV when used as antisense molecules.[4-6] This molecule has such excellent properties, for example, as high duplex

stability against complementary DNA, high inhibitory capability against various viruses, *etc.*, but has not reached the stage of clinical application yet. One of the most important reasons for this is that the mechanisms of gene regulatory function, like other antisense molecules, are not established yet so far. It is appropriate at the present stage, therefore, to unravel the antisense mechanism of oligodeoxynucleoside phosphorothioates in view of their interactions with target molecules such as DNA and RNA as well as with proteins.

To explore the above mentioned points, however, there is an unsolved high barrier that thiophosphate groups formed by the introduction of sulfur atoms into internucleotide phosphodiester linkages give rise to diastereomers and their number increases in proportion to 2^n (n: number of thiophosphate). To overcome this potential problem, a stereocontrolled solid-phase synthesis has been reported, where 5'-*O*-DMTr-nucleoside 3'-*O*-(2-thio-1,3,2-oxathiaphospholane) was taken as a chain elongation monomer. The maximum size obtained by their method, however, was at most a 7-8 mer[7] and, therefore, this method has not been used for practical purposes: Oligodeoxynucleoside phosphorothioates used currently in the clinical antisense trials are, therefore, a mixture of a number of isomers synthesized by the conventional phosphoramidite method. Considering such background, we should examine alternative ways to obtain stereocontrolled samples.

In the present study, we prepared DNA having one or two thiophosphate linkages (referred to S-oligo in this study) and separated their diastereomers by the combination of reversed phase liquid (RPLC)[8] and DEAE-ion exchange chromatography (IELC).[9] Individually separated isomers were utilized as samples.

Regarding the major subject mentioned earlier in this text, it has been reported previously[10,11] that a duplex formed between an oligodeoxynucleoside phosphorothioate and its target DNA is less stable than that formed between a pair of complementary DNA strands, and that the Sp isomer led to higher helix stability than the Rp. However, the physico-chemical property of these molecules have not been explored in detail so far. Only a few papers on the solution structure of such duplexes have been reported.[10,12] It was suggested in the papers that an oligodeoxynucleoside phosphorothioate strand in a duplex with RNA as a target is more flexible than DNA or RNA strands in their homoduplexes.[12] However, detailed studies on the stability of oligodeoxynucleoside phosphorothioate duplexes have not been carried out so far to answer the question of whether isomers with different chirality have different antisense efficiency. To assess this point, in the present study, we investigated the stability of duplexes between the isolated isomers of S-oligos and their complementary target DNA or RNA based on the melting temperature (Tm) measurements.

MATERIALS AND METHODS

Synthesis of oligonucleotides

Both DNA and S-oligos were synthesized on an automated synthesizer, Milligen/Biosearch Cyclone Plus DNA Synthesizer (Milligen, Burlington, MA, USA) by the standard phosphoramidite methods. All reagents used were purchased from Millipore. Synthesis was carried out on a 1 μmol scale. Oligodeoxynucleoside phosphoramidites were treated either with iodine or with tetraethylthiuram disulfide to generate a phosphodiester linkage and a thiophosphate linkage, respectively. Deprotection of the oligomers was performed in conc. NH_3 at 55 °C for 7 hr. After evaporation of NH_3, the oligomers were subjected to RPLC or IELC separation.[8,9]

Solid phase RNA synthesis was carried out using 5'-DMTr 3'-(cyanoethyl *N,N*-diisopropylphosphoramidites) protected with a *tert*-butyldimethylsilyl (TBDMS) group at the 2'-position (Milligen). RNA deprotection and cleavage from the support were conducted with conc. NH_3 for 7 hr. After evaporating NH_3, the TBDMS group was removed by dissolving the sample in a 1 M pyridine solution of tetrabutylammonium fluoride (TBAF) and by subsequently allowing the solution to stand overnight at room temperature. The released TBAF was removed by size exclusion column chromatography (Sephadex G-25, DNA grade, Pharmacia, Upsala, Sweden). The solution was then evaporated to dryness and the oligomers subjected to RPLC purification.

Enzymatic digestion of S-oligo

The absolute configurations of the separated S-oligo isomers were determined by specific enzymatic digestion: Nuclease P1 (Wako Pure Chemical Industries, Osaka, Japan) and snake venom phosphodiesterase (Pharmacia) specifically digest the Sp and Rp diastereomers, respectively. Calf intestine alkaline phosphodiesterase (Toyobo, Osaka Japan) was used to remove the remaining free phosphate groups.

For nuclease P1 digestion, each oligonucleotide was dissolved in a mixture of 50 mM acetate buffer (40 μL, pH 5.3), 10 mM $ZnSO_4$ (4 μL) and the enzyme (40 μL, 200 units/mL). For snake venom phosphodiesterase digestion, the same quantity of S-oligo was dissolved in a mixture of 100 mM Tris-HCl buffer (50 μL, pH 8.9), 20 mM $MgCl_2$ (5 μL) and the enzyme (20 μL, 250 units/mL). The reaction mixtures were incubated for 1 hr at 37 °C. The digestion mixtures were analyzed by RPLC employing the same conditions as used for the separation of S-oligo.

After S-oligo was digested by nuclease P1 or snake venom phosphodiesterase, the sample was treated with alkaline phosphatase. For alkaline phosphatase digestion, the sample was dissolved in 40 μL of 50 mM Tris-HCl (pH 8.0) containing 1 mM $MgCl_2$

and 6 mL of alkaline phosphatase (2 units/mL). The reaction mixture was incubated for 1 hr at 37 °C and analyzed by RPLC under the same conditions as described for the separation of S-oligo.[13-15]

UV absorption and circular dichroism spectroscopy

The extinction coefficients at 260 nm were calculated for oligonucleotides by the nearest-neighbor methods.[16] The same values were used for DNA and S-oligo with identical base sequences. Oligonucleotides were dissolved at 10 mM concentration in a phosphate buffer containing 100 mM NaCl at pH 7.0. UV absorption spectra were recorded using a UV-260 spectrophotometer (Shimadzu, Kyoto, Japan). Circular dichroism (CD) spectra were measured in the range from 210 to 320 nm with a J-720 spectropolarimeter (Jasco, Osaka, Japan) using a quartz cell (1 cm in path length) at 0 °C.

Tm measurement

All optical measurements were made on a UV-260 spectrophotometer equipped with a temperature controller SPR-5 and a temperature programmer KPC-5 (Shimadzu). Absorbance at 260 nm was recorded in 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl. All duplexes were formed as 1:1 mixtures. All samples were pre-melted at 75-80 °C to destroy secondary structures and then slowly cooled down to 0 °C. Tm measurements were initiated near 0 °C and the temperature was elevated at 0.5 °C/min until complete Tm curves were obtained (up to about 70 °C). The temperature was monitored by a thermocouple. Each melting curve is composed of at least 150 data points. The data were analyzed following the literature.[17] The melting curves were measured at least twice for each sample.

RESULTS AND DISCUSSION

Synthesis and the configuration analysis of S-oligo

In order to investigate the difference of the duplex stability between the diastereomers, we prepared dGCNsN'CG (s: thiophosphate, N, N' = A or T) and dGCsTsACG. The chain length of the oligomers is 6mer, because with this chain length of S-oligo it is possible to separate Rp/Sp isomers by RPLC and IELC and because this chain length gives enough duplex stability for spectroscopic measurements. In order to increase duplex stability, CG pairs were placed at the both ends of the oligomers: Placement of GC and CG segments on the 5' and 3' termini, respectively, avoids self complementary duplex formation. To avoid an abnormal structure, A or T was placed in the immediately flanking position of the thiophosphate linkage.

Separation of diastereomers of the prepared S-oligo was performed by RPLC and IELC. For dGCAsACG and dGCAsTCG, the two diastereomers could be separated by RPLC. On the other hand, the diastereomers of dGCAsACG and dGCAsTCG were

separated by IELC: RPLC produced poor resolution.[9] For dGCsTsACG good separation was obtained by RPLC and four diastereomers could be isolated, as depicted in Fig. 1.

To determine the absolute configuration of the thiophosphate groups of the isolated isomers, snake venom phosphodiesterase and nuclease P1 were used, and it was found that for all dGCNsN'CG, the Rp isomer eluted faster than the Sp in RPLC, which agreed with the published data.[8] In the case of dGCsTsACG, the SS and RR isomers were readily determined by specific enzymatic digestion. Since RS and SR configurations could not be determined by the conventional method, assignment was performed by coinjecting digestion products expected for RS and SR isomers (dCsT, dTsA, and dCsTsA), which were synthesized by the conventional procedure. As shown in Fig. 1, these diastereomers were found to elute in the order of RR, SR, RS, and SS.

Tm measurement for duplexes formed between S-oligo and its target DNA or RNA

As already mentioned earlier in the text, it was reported that a DNA-DNA duplex was more stable than an S-oligo-DNA duplex, and that Sp isomers formed more stable duplexes than Rp.[10,11] To see if these results are generally acceptable, Tm measurements were carried out for the duplexes produced between isolated diastereomers of dGCNsN'CG and their complementary DNA or RNA. Shown in Fig. 2 are the melting profiles obtained for the duplexes between the Rp and Sp isomers of dGCTsTCG and their complementary DNA and RNA, as well as for DNA-DNA and DNA-RNA duplexes with the same base sequence. Table 1 summarizes the Tm values obtained from the melting curves for all the possible duplexes of dGCNxN'CG (x = s or o, N, N' = A or T). The values in the table implied that the Tm values of all the duplexes formed between S-oligo strands and their target DNA or RNA were lower than those obtained for the corresponding DNA-DNA and DNA-RNA duplexes, and that Sp isomers consistently formed more stable duplexes than Rp when DNA was a target. These results agree with the data reported previously.[10,11] Additionally, it was found that the Tm difference [ΔTm = Tm(Sp)-Tm(Rp)] between the duplexes of a couple of Rp/Sp isomers was sequence-dependent. For instance, in the case of the duplex formed between dGCTsACG and its target DNA, the Tm difference was large (ΔTm = 4.2 °C) while in the case of dGCAsTCG, it was only 0.7 °C. These data indicate that choice of the base sequence influences the binding affinity of S-oligo to the target DNA. On the other hand, when RNA was used as a target, different results were obtained although duplex stability was also dependent on the base sequence. We found that for dGCTsTCG and dGCAsTCG, higher Tm values were obtained for the Rp isomers than for the Sp; ΔTm =

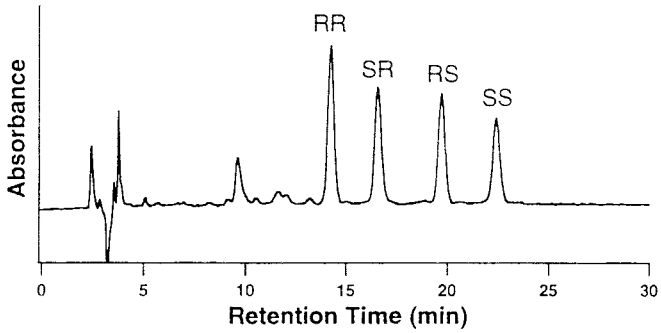

Fig. 1 RPLC separation of dGCsTsACG. Configurations of the thiophosphate linkage are marked above the peaks.
Chromatographic conditions: column, RP-18 (e) (4.6X250 mm, 4 μm); eluent, (A) 0.1 M triethylammonium acetate and (B) 50 % CH_3CN in 0.1 M triethylammonium acetate; gradient, 10-40 % B for 25 min (linear) and then 40- 100 % B for 10 min (linear); detection, 254 nm; flow rate, 0.7 mL/min.

-0.8 °C and -1.5 °C, respectively. This is the first observation that Rp isomers could form more stable duplexes than Sp.

To investigate in more detail, duplex stability was evaluated with an S-oligo having two thiophosphate groups. The sequence of S-oligo used was dGCsTsACG. The resultant Tm values obtained with its complementary DNA and RNA as a target are listed in Table 2. The data indicate that the introduction of two thiophosphate groups leads to a larger decrease in the Tm values in comparison to those of the corresponding DNA-DNA and DNA-RNA hybrids. We also compared the Tm difference between the duplexes of the isomers. For instance, when DNA was a target, the RR isomer showed the lowest Tm (13.1 °C) and the SS the highest (18.0 °C); The Tm difference between these isomers was 4.9 °C. By comparison with the ΔTm for dGCTsACG (4.2 °C), it is indicated that with an increasing the number of thiophosphate groups, the Tm difference between diastereomers becomes larger. When RNA was a target, the RR isomer had the lowest Tm (14.2 °C) and the SS the highest (16.1 °C); it should be noted that the Tm difference was 1.9 °C, which was much lower than when DNA was used as a target.

It is deduced that the influence of the chirality of the internucleoside thiophosphate on the duplex structure is almost negligible if RNA is a target. This is possibly because the structure of the S-oligo in an S-oligo-RNA duplex is flexible in comparison to a DNA strand in DNA-DNA or DNA-RNA duplexes.[12] Consequently, in the practical use of an S-oligo as an antisense medicine where RNA is a target, Rp/Sp separation may not be absolutely necessary.

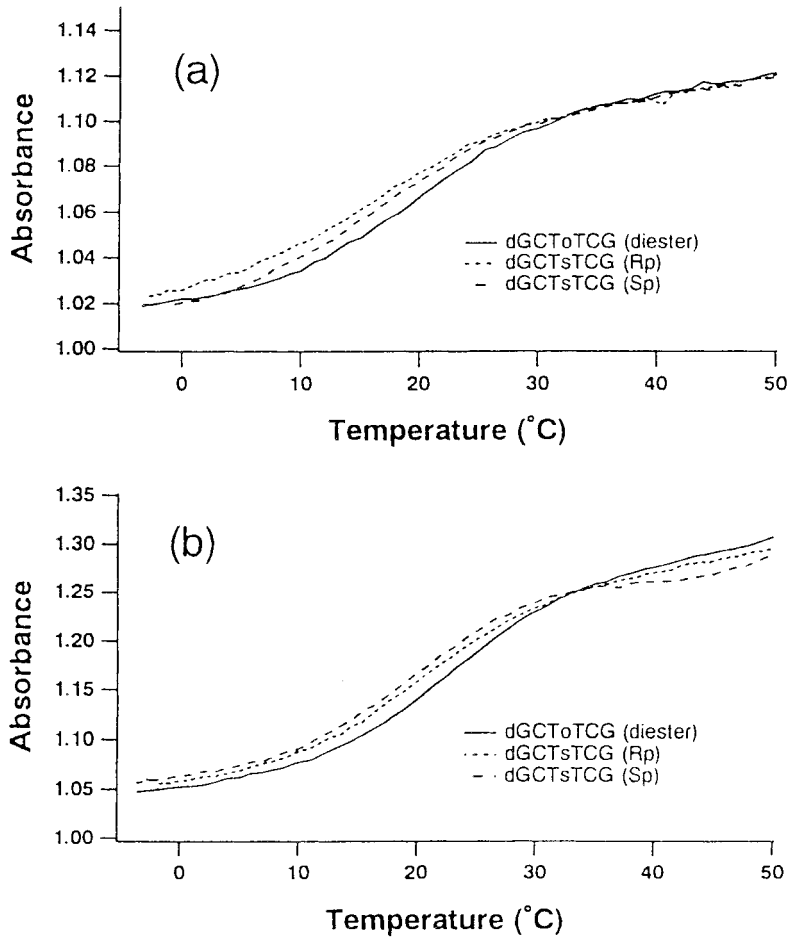

Fig. 2 Melting profiles of duplexes formed by dGCTxTCG (x = s or o) with (a) dCGAAGC and (b) rCGAAGC.
Solvent: 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl.

Table 1 Melting temperature for S-oligo duplexes[a)]

Antisense[b)]	Target	Tm (°C)			ΔTm (°C)
		diester	Rp	Sp	[Tm(Sp)-Tm(Rp)]
dGCTxTCG	dCGAAGC	20.4	16.0	17.6	1.6
dGCAxTCG	dCGATGC	25.1	22.0	22.7	0.7
dGCTxACG	dCGTAGC	20.0	14.9	19.1	4.2
dGCAxACG	dCGTTGC	25.4	22.5	24.5	2.0
dGCTxTCG	rCGAAGC	22.9	20.8	20.0	-0.8
dGCAxTCG	rCGAUGC	21.9	20.5	19.0	-1.5
dGCTxACG	rCGUAGC	20.9	17.5	18.6	1.1
dGCAxACG	rCGUUGC	17.7	15.3	16.9	1.6

a) In 10 mM phosphate buffer (pH 7.0) containing 100 mM NaCl.

b) x: phosphodiester or thiophosphate.

Table 2 Melting temperature for S-oligo duplexes [a)]

Antisense[b)]	Target	configuration	Tm (°C)
dGCxTxACG	dCGTAGC	diester	20.0
		RR	13.1
		SR	15.5
		RS	16.4
		SS	18.0
dGCxTxACG	rCGUAGC	diester	21.9
		RR	14.2
		SR	15.6
		RS	15.5
		SS	16.1

a) 10 mM phosphate buffer (pH 7.0) containing 100 mM NaCl.
b) x: phosphodiester or thiophosphate.

Analysis of structural difference of duplexes by CD measurement

The Tm difference between diastereomers becomes larger for S-oligo-DNA duplexes than S-oligo-RNA. To see the structural differences of such duplexes, we carried out CD spectroscopic measurements. Fig. 3 shows the CD spectra obtained for the duplexes [dGCTsTCG + dCGAAGC (or rCGAAGC)] at 0 °C: As a reference, the spectra produced by the duplexes [dGCTTCG + dCGAAGC (or rCGAAGC)] are also attached.

In CD spectra of hetero duplexes between DNA and RNA, generally a positive cotton effect appears at 270 nm. This effect should appear around 262 nm if an RNA-RNA duplex produces a typical A form. Also it is known that a strong negative cotton effect around 250 nm indicates a typical B form DNA-DNA duplex.[18)] Based on this data, duplexes formed with DNA as a target were analyzed to be in a typical B form conformation since they showed a strong negative cotton effect at 250 nm. On the other hand, the duplexes of S-oligo isomers with RNA were in a typical DNA-RNA hetero duplex conformation since they exhibited both a smaller negative cotton effect at 255 nm than a DNA-DNA duplex, and a positive cotton effect at 270 nm. Conclusively it is suggested that the structure of duplexes formed by S-oligo with DNA and RNA is basically similar to the structure of DNA-DNA and DNA-RNA duplexes, respectively. Small differences seen in the spectra, for example between S-oligo(Rp or Sp)-DNA and DNA-DNA, may suggest slightly different stacking structures affecting the duplex stability.

Until now, the gene regulatory ability of the antisense method with oligodeoxynucleoside phosphorothioates has been evaluated both *in vitro* and *in vivo*

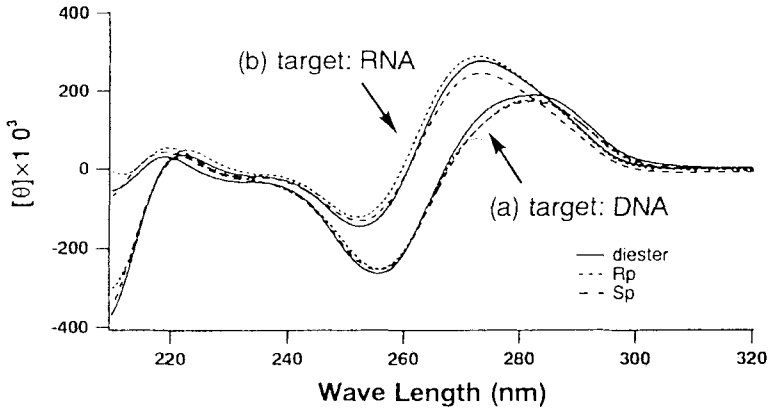

Fig. 3 CD spectra of duplexes formed by dGCTxTCG (x = s or o) with (a) dCGAAGC and (b) rCGAAGC.
Conditions: solvent, 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl; temperature, 0 °C.

only by using a mixture of diastereomers, although accurate evaluation for the effect of the Rp/Sp configuration of the thiophosphate on the duplex formation with the target RNA has been strongly required to reveal the entire mechanism of this method. In the present study, therefore, we measured Tm values of the duplexes formed by individually isolated S-oligo isomers, which are oligodeoxynucleotides having one or two thiophosphate linkages. The Tm values were obtained using DNA and RNA as a target and compared. When DNA was a target, Tm values of S-oligo duplexes changed as a function of the base sequences and large Tm difference was also observed between the Rp/Sp isomers. On the other hand, in the case of RNA as a target, such tendency became smaller, indicating that duplexes of S-oligo with RNA show different thermal behavior from those with DNA. Since RNA is a target molecule for the antisense method, it is deduced from our current results that entire Rp/Sp separation of oligonucleotide phosphorothioates may be of no major concern. It is also an important suggestion for the accurate evaluation for designing antisense molecules that Tm measurements should be carried out using RNA as a target.

ACKNOWLEDGMENT

This work was supported by Rational Drug Design Laboratories. We also wish to thank Dr. Hiroshi Ide, Professor of Kyoto Institute of Technology, for his helpful discussion.

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Received December 12, 1995
Accepted February 1, 1996